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EDITED BY

SIMON FLEXNER, M. D. PEYTON ROUS, M. D.

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CONTENTS.

No. 1, JULY 1, 1924.

PAGE

JORDAN, H. E., and SPEIDEL, C. C. The behavior of the leucocytes during coincident regeneration and thyroid-induced metamorphosis in the frog larva, with a consideration of growth factors 1

MOISE, THEODORE S., and SMITH, ARTHUR H. Diet and tissue growth. I. The regeneration of liver tissue on various adequate diets. Plates 1 and 2 13

MCMASTER, PHILIP D. Studies on the total bile. VI. The influence of diet upon the output of cholesterol in the bile ... 25

ORCUTT, MARION L. Flagellar agglutinins 43

COWDRY, E. V., and NICHOLSON, F. M. The coexistence of protozoan-like parasites and meningoencephalitis in mice. Plates 3 and 4 51

JONES, F. S. The effects of the intratracheal administration of foreign serum 63

JONES, F. S. The permeability of the lining of the lower respiratory tract for antibodies 73

BRONFENBRENNER, JACQUES J., SCHLESINGER, MONROE J., and ORR, PAUL F. Cause of immediate death by large doses of *botulinus* toxin 81

LANDSTEINER, K., and VAN DER SCHEER, JAMES. On the specificity of agglutinins and precipitins 91

WEBSTER, LESLIE T. The epidemiology of a rabbit respiratory infection. IV. Susceptibility of rabbits to spontaneous snuffles 109

WEBSTER, LESLIE T. The epidemiology of a rabbit respiratory infection. V. Experimental snuffles 117

DU NOÜY, P. LECOMTE. Surface tension of serum. IX. Time-drop and smallpox vaccination 129

DU NOÜY, P. LECOMTE. Surface tension of serum. X. On the thickness of the monomolecular layer of serum 133

No. 2, August 1, 1924.

PAGE

Aub, Joseph C., Reznikoff, Paul, and Smith, Dorothea E. Lead studies. III. The effects of lead on red blood cells. Part 1. Changes in hemolysis..... 151

Aub, Joseph C., Reznikoff, Paul, and Smith, Dorothea E. Lead studies. III. The effects of lead on red blood cells. Part 2. Surface phenomena and their physiological explanation. Plate 5..... 173

Aub, Joseph C., and Reznikoff, Paul. Lead studies. III. The effects of lead on red blood cells. Part 3. A chemical explanation of the reaction of lead with red blood cells..... 189

Smith, Arthur H., and Moise, Theodore S. Diet and tissue growth. II. The regeneration of liver tissue during nutrition on inadequate diets and fasting..... 209

Smith, Theobald. Some cultural characters of *Bacillus abortus* (Bang) with special reference to CO_2 requirements..... 219

Thompson, William P., and Meleney, Frank L. A comparative method for testing the enzymes of living hemolytic streptococci. I. Lipase..... 233

Stevens, Franklin A., and Dochez, A. R. Studies on the biology of streptococcus. III. Agglutination and absorption of agglutinin with *Streptococcus scarlatinæ*..... 253

Lewis, Paul A. The inhibitory effect of acridine on the sporogony of a coccidium (*Eimeria stiedæ*)..... 263

Morgan, Hugh J., and Neill, James M. Methemoglobin formation by sterile culture filtrates of pneumococcus..... 269

No. 3, September 1, 1924.

Rivers, Thomas M., and Tillett, William S. The lesions in rabbits experimentally infected by a virus encountered in the attempted transmission of varicella. Plates 6 to 9..... 281

Reid, Mont R. Partial occlusion of the pulmonary aorta and inferior vena cava with the metallic band. Observations on changes in the vessel wall and in the heart. Plate 10..... 289

Reid, Mont R. Partial occlusion of the aorta with silk sutures, and complete occlusion with fascial plugs. The effect of ligatures on the arterial wall. Plates 11 and 12..... 293

PAGE

HEIDELBERGER, MICHAEL, and AVERY, OSWALD T. The soluble specific substance of pneumococcus. Second paper..... 301

IONESCO-MIHAIESTI, C. Studies on the Twort-d'Hérelle phenomenon..... 317

JAFFE, HENRY L. The influence of the suprarenal gland on the thymus. I. Regeneration of the thymus following double suprarenalectomy in the rat. Plate 13..... 325

MUELLER, J. HOWARD, and TOMCSIK, JOSEPH. The chemical nature of residue antigen prepared from yeast..... 343

STILLMAN, ERNEST G. Persistence of inspired bacteria in the lungs of alcoholized mice..... 353

NAKAHARA, WARO. Effect of fatty acids on the resistance of mice to transplanted cancer..... 363

BECK, CLAUDE S., and CUTLER, ELLIOTT C. A cardiovalvulotome. Plates 14 and 15..... 375

TRASK, JAMES D., JR., and BLAKE, FRANCIS G. Observations on the presence of a toxic substance in the blood and urine of patients with scarlet fever. Plate 16..... 381

WEBSTER, LESLIE T., and PRITCHETT, IDA W. Microbic virulence and host susceptibility in paratyphoid-enteritidis infection of white mice. V. The effect of diet on host resistance..... 397

No. 4, OCTOBER 1, 1924.

NEILL, JAMES M., and AVERY, OSWALD T. Studies on oxidation and reduction by pneumococcus. VI. The oxidation of enzymes in sterile extracts of pneumococcus..... 405

NEILL, JAMES M., and AVERY, OSWALD T. Studies on oxidation and reduction by pneumococcus. VII. Enzyme activity of sterile filtrates of aerobic and anaerobic cultures of pneumococcus..... 423

MARINE, DAVID, MANLEY, O. T., and BAUMANN, EMIL J. The influence of thyroidectomy, gonadectomy, suprarenalectomy, and splenectomy on the thymus gland of rabbits. Plates 17 and 18..... 429

HITCHCOCK, CHARLES H. Classification of the hemolytic streptococci by the precipitin reaction..... 445

PAGE

SHIBLEY, GERALD S. Studies in agglutination. II. The relationship of reduction of electrical charge to specific bacterial agglutination 453

ROBERTSON, OSWALD H., and SIA, RICHARD H. P. Studies on pneumococcus growth inhibition. III. The influence of specific antipneumococcus serum on the growth-inhibitory and bactericidal action of normal serum-leucocyte mixtures. 467

ROBERTSON, OSWALD H., WOO, SHUTAI T., and CHEER, SHEO NAN. Studies on pneumococcus growth inhibition. IV. A simplified agitator for growth inhibition tests with serum-leucocyte mixtures; and certain modifications in the technique of the test. Plate 19 487

STEVENS, FRANKLIN A., and DOCHEZ, A. R. Studies on the biology of streptococcus. IV. The occurrence of *Streptococcus scarlatinæ* in convalescence and in the complications of scarlet fever 493

LEWIS, PAUL A., and LOOMIS, DOROTHY. Allergic irritability. The formation of anti-sheep hemolytic amboceptor in the normal and tuberculous guinea pig 503

CLARK, HARRY, and STURM, ERNEST. The production of persistent alopecia in rabbits by Roentgen radiation; a study of the minimum dose required and the consistency of the reaction 517

MILLER, C. PHILIP, JR. Attempts to transmit rheumatic fever to rabbits and guinea pigs 525

MILLER, C. PHILIP, JR. Spontaneous interstitial myocarditis in rabbits. Plates 20 and 21 543

REIMANN, HOBART A. The blood platelets in pneumococcus infections 553

STILLMAN, ERNEST G. Production of immunity in mice by inhalation of pneumococci 567

No. 5, NOVEMBER 1, 1924.

HITCHCOCK, CHARLES H. Precipitation and complement fixation reactions with residue antigens in the non-hemolytic streptococcus group 575

PAGE

BROWN, WADE H., PEARCE, LOUISE, and VAN ALLEN, CHESTER M. Studies based on a malignant tumor of the rabbit. VI. Variations in growth and malignancy of transplanted tumors. Part 1. Results of transplantation for the first twenty generations 583

PEARCE, LOUISE, BROWN, WADE H., and VAN ALLEN, CHESTER M. Studies based on a malignant tumor of the rabbit. VI. Variations in growth and malignancy of transplanted tumors. Part 2. Factors influencing the results of serial transplantation 603

JAFFE, HENRY L. The influence of the suprarenal gland on the thymus. II. Direct evidence of regeneration of the involuted thymus following double suprarenalectomy in the rat. Plate 22 619

ORCUTT, MARION L. The effect of heat on flagellar and somatic agglutination 627

MUDD, STUART, and MUDD, EMILY B. H. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. Plates 23 and 24 633

MUDD, STUART, and MUDD, EMILY B. H. Certain interfacial tension relations and the behavior of bacteria in films 647

NUKADA, S., and MATSUZAKI, T. Tissue resistance and the cause of permanent acquired immunity 661

THJÖTTA, TH. On the nature of the heat-labile substance ("V") required for the growth of the bacillus of Pfeiffer 671

BLOOMFIELD, ARTHUR L., and FELTY, AUGUSTUS R. The relation of dose of bacteria to group infection 679

FELTY, AUGUSTUS R., and BLOOMFIELD, ARTHUR L. The relation of vegetative activity of bacteria to pathogenicity 703

No. 6, DECEMBER 1, 1924.

PAPPENHEIMER, ALWIN M., and LARIMORE, LOUISE D. The occurrence of gastric lesions in rats. Their relation to dietary deficiency and hair ingestion. Plates 25 to 28 719

STILLMAN, ERNEST G., and BRANCH, ARNOLD. Experimental production of pneumococcus pneumonia in mice by the inhalation method 733

PAGE

BRANCH, ARNOLD, and STILLMAN, ERNEST G. Pathology of experimental pneumococcus pneumonia in mice. Plates 29 to 31 743

JAFFE, HENRY L. The influence of the suprarenal gland on the thymus. III. Stimulation of the growth of the thymus gland following double suprarenalectomy in young rats. Plate 32 753

PARKER, JULIA T. The production of an exotoxin by certain strains of *Staphylococcus aureus*. Plate 33 761

MILLER, C. PHILIP, JR., ANDREWES, C. H., and SWIFT, HOMER F. A filterable virus infection of rabbits. I. Its occurrence in animals inoculated with rheumatic fever material. Plates 34 and 35 773

ANDREWES, C. H., and MILLER, C. PHILIP, JR. A filterable virus infection of rabbits. II. Its occurrence in apparently normal rabbits 789

DRURY, DOUGLAS R. Studies on the total bile. VII. Conditions influencing the calcium content of the bile 797

HARRIS, WILLIAM H., and DUVAL, CHARLES W. Studies upon the etiology of dengue fever. I. Experimental transmission to the lower animal 817

DUVAL, CHARLES W., and HARRIS, WILLIAM H. Studies upon the etiology of dengue fever. II. Cultivation and nature of the virus. Plates 36 to 38 835

SABIN, F. R., AUSTRIAN, C. R., CUNNINGHAM, R. S., and DOAN, C. A. Studies on the maturation of myeloblasts into myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia. Plates 39 and 40. 845

INDEX TO VOLUME XL 873

THE BEHAVIOR OF THE LEUCOCYTES DURING COINCIDENT REGENERATION AND THYROID-INDUCED METAMORPHOSIS IN THE FROG LARVA, WITH A CONSIDERATION OF GROWTH FACTORS.

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I.

INTRODUCTION.

Various aspects of the problem of growth stimulation of tissues have been emphasized recently. From experiments with tissue cultures Carrel (1) has obtained results showing that leucocytes have a growth-promoting function, that they give off substances which stimulate the growth of adjacent cells. These growth-promoting substances he has named trephones (2). On the basis of the specific differential distribution of leucocytes in thyroid-treated tadpoles, the writers (3) have concluded that, if leucocytes have a growth-promoting function, this function is probably to be ascribed to the lymphocytes; that the granulocytes, on the contrary, probably give off substances that aid in the breaking down of tissues.

In a recent series of studies on cancer, Burrows (4) finds the following conditions favorable and probably essential to growth: a relatively crowded condition of cells, a relatively reduced condition of vascularization, and absence of intercellular materials. These spatial conditions, he believes, favor the accumulation of certain products of normal cellular metabolism. Among these metabolites is to be found the fundamental stimulating agent causing increase in growth rate of adjacent cells. On the other hand, Burrows finds that a loose arrangement of cells, much vascularization, and the presence of intercellular materials are conditions which prevent the accumulation of cellular metabolites, and are, therefore, conditions which inhibit growth.

In a number of earlier studies upon amphibians the writers have discussed the rôle of various types of blood cells both in relation to changes in metabolic

rate and in relation to local processes of progressive and regressive change (Jordan and Speidel (3, 5, 6)). Some of these conclusions may be summarized as follows:

1. Granulocyte production is stimulated by the products of tissue lysis (proteolysis). Eosinophilic granulocytes are stimulated by that type of proteolysis occurring normally in the *intestine* of a frog larva undergoing thyroid-induced metamorphosis, and occurring pathologically in infections by certain worms and protozoa. Neutrophilic granulocytes are stimulated by that type of tissue lysis occurring normally in the *tail* of a frog larva undergoing thyroid-induced metamorphosis, and occurring pathologically in infections by many types of bacteria. Both eosinophils and neutrophils are believed to exert a lytic effect upon the adjacent tissues, thus preparing them for phagocytosis by the macrophages (monocytes) or for elimination by a sloughing process.

2. The lymphocytes, functioning in the frog as blood mother cells for the erythrocytes, the granulocytes, the thrombocytes, and the macrophages, are stimulated by the various factors that underlie the normal production of the definitive types of blood cells. These would include prominently the stimuli of proteolysis and of increased production of cellular metabolites, especially carbon dioxide. The lymphocyte is also the particular type of leucocyte of chief importance in the growth-promoting effect upon adjacent tissues.

3. Erythrocyte production is stimulated by some product of normal cellular metabolism, probably carbon dioxide.

4. Macrophage production is called forth by the residue of tissue autolysis.

5. Thrombocytes are interpreted as abortive erythrocytes. They develop only from the smallest lymphocyte progenitors.

6. Basophilic granulocytes are interpreted for the most part, if not entirely, as immature stages in the differentiation of eosinophils.

The tissues of the frog larva offer excellent material for the study of blood cells in relation to progressive and regressive changes. In a tadpole in which metamorphosis is initiated by thyroid administration, certain regions, *e.g.* tail, intestine, undergo rapid regressive changes; other regions, *e.g.* limbs, undergo rapid progressive changes. It occurred to the writers that coincident progressive and regressive changes might be readily induced experimentally, by simultaneous thyroid administration and removal of a part of the tail. The removal of a portion of the tail of a normal tadpole is quickly followed by regeneration of the lost part. This means an acceleration of the growth mechanism in that region. Treatment of the animal with thyroid extract not only inhibits growth in the tail, but it initiates the process of degeneration. In the conflict between the mechanism

for regeneration and the mechanism for degeneration our purpose was first, to obtain data regarding the behavior of the leucocytes, and, second, to add further data regarding the conditions determining growth.

Some experiments were also performed on the limbs, consisting of simultaneous thyroid administration and removal of a portion of the hind limb. In this case it is clear that both reactions, that of growth of the hind limb and that of regeneration of the lost part, are of the progressive kind.

After our experiments had been completed it was found that Romeis (7) had made a study of the effect upon regeneration of thyroid and thymus. His study, however, is purely from the macroscopic viewpoint, and in no way touches our results upon the behavior of the blood cells or the factors concerned in growth.

EXPERIMENTS AND OBSERVATIONS.

The frog larvæ used in the experiments, forty-eight in number, were collected on December 4, and the experiments started immediately. The size, as indicated by the total body length, ranged from 27 to 80 mm., most of the tadpoles being of intermediate size. Approximately one-half the tail was removed, a hot knife being used. Thyroid administration was accomplished by placing some dried extract of sheep thyroid in the water with the tadpoles.

The animals were divided into seven lots as follows, each lot containing large, medium sized, and small animals:

Lot 1.—Eight tadpoles (Nos. 309–316). Kept in water with thyroid extract starting Dec. 4. One-half of tail removed 2 days later, Dec. 6. Four died; Dec. 8, 11, 12, 13, respectively. The other four were killed; Dec. 8, 11, 12 (two).

Lot 2.—Ten tadpoles (Nos. 319–328). Kept in water with thyroid extract starting Dec. 4. One-half tail removed the same day, Dec. 4. Three died; Dec. 11, 13 (two). The other seven were killed; Dec. 6, 8 (two), 10 (two), 11, 13.

Lot 3.—Ten tadpoles (Nos. 329–338). One-half tail removed on Dec. 4. Kept in water with thyroid extract starting 2 days later, Dec. 6. Two died; Dec. 11, 13. The other eight were killed; Dec. 7, 8, 9, 10, 11, 12, 13 (two).

Lot 4.—Five tadpoles (Nos. 339–343). Control animals. Tail left intact. Kept in water with thyroid extract starting Dec. 4. Two died; Dec. 11. The other three were killed; Dec. 6, 8, 10.

Lot 5.—Five tadpoles (Nos. 344–348). Control animals. Kept in water without thyroid extract. One-half tail removed Dec. 4. No deaths. The five animals were killed; Dec. 6, 8, 10 (two), 13.

Lot 6.—Five tadpoles (Nos. 349–353). Control animals. Kept in water without thyroid extract. Tail left intact. No deaths. The five animals were killed; Dec. 6, 9, 12, 13 (two).

Lot X.—Five tadpoles (Nos. 355–359). Kept in water with thyroid extract starting Dec. 4. One-half tail and a variable portion of both hind limbs removed the same day, Dec. 4. Three died; Dec. 11, 13 (two). The other two were killed; Dec. 7, 11.

In addition a few tadpoles from another lot were used for a study of the early changes following tail removal, at the end of 7 hours, 18 hours, and 43 hours. Direct observations were also made on the living regenerating tail at many stages in its growth period.

Mortality.—The mortality was high in the lots that were subjected to thyroid treatment. The operation of tail removal apparently somewhat decreases the ability of the animal to withstand the thyroid treatment, and reciprocally the thyroid administration decreases the ability of the animal to recover from the operation.

Regeneration.—Regulation of the tissues in the cut region quickly takes place. An almost immediate cessation of circulation in the vicinity of the cut region occurs. The cut muscles retract somewhat, carrying skin and connective tissue with them, thus bringing about protrusion of the notochord. Autolysis in the cut region is initiated. In a normal tadpole, untreated with thyroid 7 hours after the tail removal, there is a thin epidermis of two cell layers completely covering the cut. After the first few days of the experiment it was obvious from macroscopic observation that the rate and amount of regeneration were greatest in Lot 5 (tadpoles which were fed no thyroid), next greatest in Lot 3 (tadpoles which were fed thyroid 2 days after the removal of the tail, next greatest in Lot 2 (tadpoles which were fed thyroid beginning the same day that the removal of the tail occurred), and least in Lot 1 (tadpoles which were fed thyroid beginning 2 days before the removal of the tail). Microscopic examination of the tissues in the regenerating zone confirmed these observations. In absolute amount new growth took place to the extent of about 5 mm. in a representative individual of Lot 5 in 9 days. In Lot 1, there was regulation of the cut surface, but almost no new growth.

In all cases the regeneration consisted chiefly of new epithelium, connective tissue, and to a less extent notochord. Muscle regeneration was noted in late stages, especially in Lot 5.

Vascularity of Regenerating Zone.—A comparison of the degree of vascularization among the first five lots revealed that it was greatest in Lot 1, next in Lot 2, next in Lot 3, and least in Lot 5. It is furthermore apparent that in Lots 1, 2, and 3, all thyroid-treated, the vascularity increases with time. In Lot 5 the most rapidly growing portion, the tip, remains the least vascularized. The other portions, with the slowing up of growth and the advance of differentiation, become progressively characterized by increase in vascularization.

Crowding of Cells in Regenerating Zone.—The mesenchyme cells were especially active in all animals in which regeneration was occurring. A comparison of sections revealed that they were most closely crowded in Lot 5, next in Lot 3, next in Lot 2, and least in Lot 1. To a less extent similar conditions obtained in regard to the epithelial cells and the cells concerned in the regeneration of the notochord.

The Leucocytes of Regenerating Zone.—The types of leucocytes that were most conspicuously present soon after the tail removal were neutrophils, and to a less extent lymphocytes and early stages in the process of differentiation of erythrocytes from lymphoid hemoblasts. In those animals not treated with thyroid (Lot 5) the leucocytes became less numerous with the advance of the regenerative process and the blood picture returned to a more normal condition. This was clearly the condition after 6 days in one animal in which about 3.5 mm. of new tail had been regenerated. In the thyroid-treated individuals the vascularity is somewhat greater at the end of 2 days, with the same types of leucocytes in evidence; neutrophils, lymphocytes, and young red cells. In later stages, with the great increase in vascularity, other types of leucocytes become abundant, especially the macrophages. There may also be seen a number of hemoblasts, connective tissue basophils, and a few eosinophils. These basophils are apparently early stages in the development of the eosinophils, before the granules have fully "ripened." The macrophages are the chief agents, in the removal of tissue débris, being especially conspicuous in the transportation of pigment. A few eosinophils from the blood vessels may appear in the later stages.

The Epithelium.—As has already been mentioned the epidermis plays the chief rôle in closing over the wound, both surface and underlying layers taking part in the process. Within 7 hours a thin epidermal membrane has spread over practically the entire cut surface.

In the stratified epithelium of the normal tadpole the surface layer of cuboidal cells is rather sharply demarcated from the several subjacent layers. The cells of the underlying layers are usually characterized by the presence of conspicuous coarse threads of mitochondrial nature. These are never seen in the surface layer. In newly regenerated skin, both in normal and thyroid-treated animals, these mitochondrial threads are not present. After about a week or so a few threads may make their appearance. In the hind limbs, with the rapid growth following thyroid administration, the mitochondria disappear almost entirely.

In each of the cells of the surface layer there is present both in normal and thyroid-treated animals a definite crescentic condensation of cytoplasm located always on the proximal side of the nucleus. This body or apparatus stains somewhat more deeply than the rest of the cytoplasm. It may become quite prominent in the cells of the regenerated zone. It appears to represent a remnant of the mitochondria. At the distal surface of the cell may be seen a number of indistinct granules, presumably degenerating pigment granules. The complete history and significance of these cytoplasmic structures in the epithelial cells of tail and limbs will be considered in detail in a separate paper.

Miscellaneous.—Lot 3 affords excellent examples showing the operation of the conflicting processes of regeneration and degeneration. The regenerative impetus gained by the removal of tail 2 days before thyroid feeding persists for several days at least before checked by the thyroid. Once the thyroid extract does take effect, however, the rapidly growing region undergoes more rapid degenerative changes than do the more stable regions outside of the regenerating zone.

The individuals of Lot X present merely a typical thyroid picture in the limbs, possibly somewhat accentuated. The characteristic lymphocyte layer is present beneath the epidermis. A few neutrophils may also be seen in the epidermis.

A comparison of the epidermis of tail and limb shows some sloughing in both regions and some multiplication of the basal cells; both processes, however, being more pronounced in the limbs. In later stages there appears on the surface in both regions a dense membrane, consisting of a single layer of squamous cells. The suggestion presents itself that this membrane is to be interpreted in terms of a protective reaction against the thyroid in the water.

INTERPRETATION AND DISCUSSION.

The Leucocytes.—The neutrophil is quite conspicuous in all of the experimental animals. Found chiefly in the dermis and underlying tissues, its presence is interpreted as associated with proteolysis. Its function is conceived to be chiefly that of giving off a secretion which furthers the process of tissue lysis. In this manner it acts as a forerunner of the macrophage, preparing tissues undergoing degeneration for their removal by the macrophages. A slight amount of phagocytosis of very small particles may also be accomplished now and then by the neutrophils, as shown by injection experiments with India ink, but there is little or no evidence for this in the process of tail degeneration. Since the neutrophils are present especially in the tail of all thyroid-treated animals, in which degeneration has been initiated, it is hardly likely that these leucocytes have any growth-promoting function.

Not much additional light is thrown by these experiments upon the function of the lymphocyte. The lymphocytes are present in the process of regeneration of the lost portion of the tail. They are not present, however, in sufficiently great numbers to warrant the conclusion that they are of prime importance in aiding the new tail growth. This condition is in direct contrast with the condition in the limbs of thyroid-treated animals where they are present in great numbers. Doubtless those present in the regenerating tail have some growth-promoting function, but this activity must be small compared to their corresponding activity in the limbs of thyroid-treated animals.

Zeleny (8) has concluded that regeneration in the tadpole tail is centrally controlled. His data may be supplemented by our observation that the leucocytes have no important local effect in aiding and maintaining the process. Carrel (2) recognizes that regeneration may

be brought about by various mechanisms; that leucocytic trephones may not be necessary in all cases for bringing about the new growth. Here the stimulation might be initiated by substances set free from disintegrating muscle cells.

The eosinophil is absent from the tail almost entirely in these experiments. A few may appear in the later stages. That this absence is not due to a scarcity of eosinophils in the entire animal is shown by an examination of the hemopoietic centers. Eosinophils are produced in great numbers in the intertubular regions of the kidney. Large numbers of eosinophils are attracted to the intestinal region, presumably by the particular type of tissue lysis and the sloughing process occurring there. This interesting differential distribution of eosinophils and neutrophils to the intestine and tail has been noted by us before (3). Both cells are believed to function in much the same way, *i.e.* to give off a secretion which causes, or at least aids, regressive change (proteolytic change). Until more light is thrown upon the exact function of these cells it can only be surmised that the eosinophil reacts to certain types of tissue lysis, the neutrophil to certain other types.

Macrophages are very numerous in the later stages in the thyroid-treated animals. This type of cell is the chief agent for the removal of pigment and other tissue débris produced by the primary degenerative processes. Hemoblasts of varying sizes appear in the tail after thyroid treatment in somewhat more than normal numbers. These hemoblasts are not localized in the tail any more than in other regions of the body. Their presence is merely a part of the general response of the body tissues to meet the tremendous demand for erythrocytes for the maintenance of the new high level of metabolism induced by the thyroid administration.

A number of basophilic granulocytes, evidently of connective tissue origin, make their appearance in later stages. The significance of their presence is not fully understood but there is evidence that they may differentiate into eosinophils. They are provisionally interpreted as precursors of eosinophils. The suggestion presents itself that the connective tissue has been stimulated to give rise to eosinophils in the tail, since this region may suffer from lack of these cells, with the strong competitive attraction for eosinophils by the intestine.

Growth Stimulation.—Our observations are of interest in connection with the recent views of Burrows (1923 (4)) on the problem of the stimulation of cells to growth. As has already been pointed out his conclusion is that the spatial conditions favorable for the stimulation of growth of cells are (1) a relatively crowded condition, (2) a relatively reduced blood supply, (3) a relative lack of intercellular substances. The fundamental stimulating agent he believes to be something given off by cells in the normal process of metabolism. A crowded condition with reduced blood supply affords a favorable basis for the accumulation of this stimulating substance. A looser relation of cells with greater vascularity, on the contrary, makes for a removal of this stimulating substance with resultant decrease in growth stimulation.

Our findings harmonize well with this conception. There is least vascularity and greatest crowding in the animals of Lot 5, those in which regeneration was most rapid. Next in order as regards amount of regeneration comes Lot 3, with somewhat greater vascularity and somewhat less crowding; then Lot 2, and Lot 1. In Lot 1, with least regeneration, the vascularity is greatest and the crowding of cells least. It is also of interest that in Lot 5 (not treated with thyroid) the vascularity varies somewhat in the different regions of the zone of regeneration. At the very actively growing tip the vascularity is least and remains so. At the ventral and dorsal fin expansions near the place of the original cut the vascularity becomes fairly normal; as great as before the cut. *Growth* here largely ceases and *differentiation* sets in. Relatively little vascularity then, is associated with growth; somewhat greater vascularity with differentiation and cessation of growth. In Lots 1, 2, and 3 the vascularity increases with time, correlated with decrease in regenerative activity. Our observations from these four lots show that there is a regular graded series of growth reactions, the amount of growth being correlated directly with the degree of crowding and inversely with the degree of vascularization. This supplements upon experimental animal material the results of Burrows on tissue cultures.

It may be noted also that in the limbs of a tadpole under thyroid treatment this correlation is not always so close. A few days after the thyroid administration the limbs become tremendously vascular-

ized, though at the same time rapid growth processes are taking place. At this stage the opposing conditions of great vascularity and crowding of cells obtain.[1] In later stages, the vascularity becomes proportionately less.

One other point may be mentioned. The cells taking part in the regenerative process following removal of the tail are functioning at a higher metabolic rate presumably than those outside the regenerating zone. The addition of thyroid should, then, according to the work of Child (9) affect the region of new growth more readily than other more stable regions. Microscopic examination shows this to be the actual case.

SUMMARY.

1. Coincident progressive and regressive changes are induced in the tail of the frog larva by simultaneous thyroid administration and removal of a portion of the tail. The rate of growth of the regenerating tail is greatest in animals not treated with thyroid, next greatest in animals treated with thyroid 2 days after tail removal, somewhat less in animals treated with thyroid simultaneously with tail removal, and least in those treated with thyroid 2 days before removal of the tail.

2. The leucocytes chiefly concerned are neutrophilic granulocytes, lymphocytes, hemoblasts, and lymphoid phagocytes (monocytes, macrophages). The neutrophils further tissue lysis, the lymphoid phagocytes remove tissue débris. The lymphocytes are apparently not numerous enough to exert any important growth-promoting influence. The hemoblasts represent merely a part of the general reaction to thyroid treatment. Eosinophils are absent except for a few that appear in the late stages. Basophils of mesenchymal origin are present in the later stages of thyroid-treated animals. These appear to differentiate further into eosinophils.

3. The rate and amount of growth are correlated directly with the degree of crowding of cells, and inversely with the degree of vascularity. The greatest crowding and least vascularity occur in animals

[1] Even here, however, most rapid growth occurs in the non-vascular and the relatively less vascular areas of the limbs, that is, in the epithelium, and the axial preosseous and premuscular regions, respectively.

not treated with thyroid, somewhat less crowding and greater vascularity in those treated with thyroid 2 days after tail removal, still less crowding and somewhat greater vascularity in those treated with thyroid at the time of tail removal, and finally, least crowding and greatest vascularity occur in animals treated with thyroid 2 days before tail removal. These results support the views of Burrows (4) upon the mechanism of growth stimulation of cells.

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DIET AND TISSUE GROWTH.

I. The Regeneration of Liver Tissue on Various Adequate Diets.*

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Plates 1 and 2.

(Received for publication, February 19, 1924.)

A consideration of the problem of growth brings out certain analogies between this phenomenon and tissue repair. Structural increase is common to both processes and it seems reasonable to suppose that the optimal nutritive conditions for the enlargement of the body as a whole are similar to those necessary for the reconstruction of one of its constituent parts. It also seems probable that the processes of repair may be controlled, just as growth is, by certain dietary factors. This is particularly interesting from a practical point of view as the determination of the optimum conditions for the rapid healing of wounds is one of the ideals in surgery. Conversely, a study of diet and tissue regeneration might also throw valuable sidelights on growth. The present investigation is concerned with the effect of various adequate diets on the reparative process in the liver.

It is obvious at the outset that any effect brought about by adjustment of dietary factors will not be a sudden one. The striking characteristic of practically all deficiency lesions or conditions is that the onset is gradual, hence very likely to escape notice. In this type of experiment, therefore, it is desirable to establish the animal definitely in nutritive equilibrium on the ration in question before producing the tissue injury. This involves, in turn, a thorough knowledge not only of what constitutes an adequate diet but also of the optimum relationship of the various factors therein. A proper appreciation of these facts is possible only as a result of contributions to the science of nutrition made during the last decade. It is of value, therefore, to repeat experiments which have been made on this point but

* Part of the expenses of this investigation was defrayed from the Russell H. Chittenden Research Fund.

under controlled conditions using synthetic diets each factor of which can be evaluated.

The effect of diet on healing of skin wounds in dogs was brought out by Clark (1919). In these experiments it was found that changing from a fat to a protein diet increased the rate of healing mainly by decreasing the length of the quiescent period. The diets used were extreme, and in no sense adequate, and the number of cases was small. Opie and Alford (1914) studied the effect of carbohydrate diet (oats and sugar), protein diet (meat), and fat diet (beef suet) on hepatic necrosis in rats, but here, too, the diets were inadequate and the length of time on the various rations was short (4 days). They concluded that the resistance to chloroform poisoning was greater on the carbohydrate than on the fat diet. Davis and Whipple (1919, *a*) (1919, *b*) and Davis, Hall, and Whipple (1919) report that carbohydrate favors regeneration of liver tissue in dogs after chloroform necroses but that starvation and fat diet are decidedly unfavorable to repair. The carbohydrate diet consisted of cane-sugar alone or with rice and potatoes; the fat diet contained butter, lard, fat meat, and cotton seed oil, mixtures which would suffice neither for growth nor probably for maintenance for any length of time. Brain, beef heart, liver, or kidney was fed at different times but in all these experiments no effort seems to have been made to accustom the animal thoroughly to the diet before giving the chloroform. According to these investigators the increased nitrogen loss observed after chloroform necrosis is markedly reduced by feeding sugar and this suggests to them that here is an elite example of the protein-sparing action of carbohydrates, an idea previously proposed by Opie (1914).

EXPERIMENTAL.

The plan of the present experiments was to produce a standard severe tissue destruction in animals which had been fed definite diets. At uniform intervals after the injection the animals were killed and the comparative rate of regeneration of tissue observed histologically. There were four diets: a normal balanced diet, a high fat diet, a high carbohydrate diet, and a high protein diet. The animals (white rats) were placed on these rations soon after weaning and allowed to grow to 80 to 100 gm. when the tissue injury was made. The demand on the organism was thus doubly severe, for tissue repair took place during the period of most rapid growth.

The albino rat was used for two reasons. First, the growth rate of these animals has been standardized through many carefully controlled experiments. The activity of the rat under given conditions can thus be predicted and those individuals not conforming to the usual behavior may be discarded. Second, the small size of the animal enables large numbers of individuals to be cared for in relatively small space and, what is more important, the food intake is small enough to permit feeding materials more or less difficult to prepare, such as

certain pure proteins or amino acids. After some preliminary work using shorter intervals it was decided to examine the livers of the rats at 24 hour intervals from 24 hours after the administration of chloroform to 144 hours after. At least four animals were used at every interval on every diet to rule out the individuality factor. They were killed by asphyxiation with artificial illuminating gas and the livers immediately removed and fixed. The technique of feeding, weighing, and caring for the animals was that outlined by Ferry (1919–20).

The composition of the diets used is as follows:

Percentage composition.		Calories per kilo of food.	Apportionment of total calories.	
Standard balanced diet.				
	per cent			*per cent*
Casein	18	738	Protein	13.8
Starch	51	2,091	Carbohydrate	39.2
Lard (or beef suet)	22	2,046	Fat	47.0
Cod liver oil	5	465		
Salts*	4			
		5,340		
High protein diet.				
Casein	80	3,280	Protein	68.7
Lard (or suet)	12	1,488	Fat	31.3
Cod liver oil	4			
Salts	4			
		4,768		
High fat diet.				
Casein	25	1,025	Protein	13.8
Lard (or suet)	65	6,417	Fat	86.2
Cod liver oil	4			
Salts	6			
		7,442		
High carbohydrate diet.†				
Casein	14	514	Protein	13.8
Starch	79	3,239	Carbohydrate	77.8
Cod liver oil	3.8	353	Fat	8.4
Salts	3.2			
		4,166		

* The salt mixture used was that of Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557).

† The high carbohydrate food was made up as a stiff paste with the weighed amount of starch and boiling water. It was then cooled and the other ingredients stirred in. The moist food was spread in thin layers and dried to a crisp cracker. It was found necessary to do this to prevent scattering of food. Also the high percentage of raw starch induced diarrhea in some of the rats. On the high fat diet the growth was somewhat subnormal but on the other diets normal growth was obtained.

Beef suet which had been melted and freed from water was substituted for lard during the summer in order to maintain a firmer consistency of the paste foods. Vitamin B was provided in 100 mg. tablets of dried yeast fed apart from the food.[1] On diets of such widely different energy value it is desirable to adjust the protein and salts to the total calories so that lack of neither of these food factors will disturb the normal growth (see Smith and Carey (1923)).

In these experiments we have used the liver merely as a tissue in which a fairly standard injury can be produced. We were not interested in this organ *per se*.

The chloroform was dissolved in sterile mineral oil and injected subcutaneously. This method was employed because the quantity could be measured accurately and because the chloroform was slowly absorbed when given with the oil. The dosage was so adjusted that the total volume of injected fluid was always 1 cc. Since we were interested primarily in survivals and, at the same time, maximum liver damage, it was necessary to determine the maximum non-lethal dose of chloroform on each of the various diets. In order to obtain a valid index of the toxicity, it is important to use only fresh chloroform. In most of the present experiments the sample of Squibb's anesthesia chloroform was redistilled and the fraction boiling between 61° and 62°C., was used within an hour.

Results.

Toxicity.—The dose of chloroform after which the majority of rats on the standard balanced ration survived was 1.5 cc. per kilo of body weight. Opie (1914) stated that 2 cc. per kilo failed to kill his rats and in some cases he gave three times that amount, but in the present experiments when 2 cc. were given, too few rats on this diet survived for our purpose. 1.5 cc. per kilo were used for the rats on the high carbohydrate diet likewise, but it seems from our data that on this latter diet the toxicity was distinctly less than on the standard balanced ration. We have not observed a greater toxicity on high protein diet than on high carbohydrate food as stated by Opie and Alford (1915). On the other hand the maximum non-lethal dose administered in our routine experiments was 2.5 cc. per kilo of body weight. It is worthy of notice that in these animals and even in a few on the high protein diet surviving 3 cc. per kilo the extent of initial necrosis is distinctly less than that on other diets after the maximum

[1] This dosage had previously been found to furnish vitamin B adequate for normal growth over the range in size involved in these experiments.

non-lethal dose. On the high fat ration the toxicity was definitely greater and the dose of chloroform had to be reduced to 1.0 cc. per kilo in order to secure enough survivals for subsequent examination. With all the above doses of chloroform definite comparable degrees of liver necrosis were observed when the animals were killed from 24 to 36 hours after the injection, *i.e.*, before repair process had set in. It appears from our experiments that the toxicity of chloroform varies with the diet in the following order of decreasing susceptibility: high fat > standard > high carbohydrate > high protein diets.

Repair of the liver. Standard Diet.—The necrosis appears at its maximum at about 24 hours after the injection, at which period there is an extensive hyaline necrosis around the central veins involving from two-thirds to four-fifths of each lobule (Fig. 1). The necrosis may involve all portions of the liver lobule except circular areas of varying size immediately surrounding the larger portal vessels. The destruction of the parenchymal cells is uniform and complete. There may be a narrow rim of liver cells, with pale vacuolated cytoplasm, indistinct cell outlines and pycnotic nuclei separating the necrotic from the living tissues. In a few instances there is a considerable amount of fat with no evident liver cells in the central three-fourths of each lobule.

The central vessels and the surrounding capillaries are distended with red blood cells and in some instances there are hemorrhages of varying extent in the necrotic areas.

At this period there is no evidence of activity either by infiltration of leucocytes or by regeneration of the liver cells.

In the 48 hour period the picture has changed markedly. The process of repair is initiated by an early mobilization of leucocytes and clearing away of the necrotic cellular debris coincident with active regeneration of the hepatic parenchyma by a proliferation of the uninjured liver cells at the periphery of the lobule. The degree of repair varies considerably and while the most conspicuous feature is in some instances, the clearing away of the cellular debris, in others the predominant finding is active regeneration of the hepatic cells as shown by numerous mitotic figures. In some areas large liver cells containing two nuclei are seen.

The mitotic figures are rarely seen at the margin of the areas of complete necrosis but are much more numerous in the deeper layers of the surviving liver cells.

In some sections in the 72 hour period there is evidence of very active and rapid regeneration (Fig. 2). The sections show very large numbers of mitotic figures and the injury has been repaired to a considerable extent. The largest remaining unhealed areas are about one-third of the size of the liver lobule. The cellular debris has been completely cleared away. There is no evidence of connective tissue proliferation. In some instances only slight remains of the injury are evident.

In the 96 hour sections nothing remains of the necrotic area. The cells are quite granular and their arrangement in columns is somewhat less regular than in the normal liver. There is no evidence of connective tissue proliferation.

In the 120 and 144 hour sections the liver is histologically normal with no evidence whatever of a recent injury. The liver could not be distinguished from a section taken from a normal animal, except for less distinct cell columns and for fatty accumulations in the central zones of some lobules.

In each period there is considerable variation in the activity and degree of repair.

High Carbohydrate Diet.—The extent of the necrosis is somewhat less than is seen on the standard diet. In the 24 and 48 hour sections, hemorrhages into the necrotic areas are conspicuous. The evidence of regeneration, in the 48 hour period, is less marked than that observed on standard diet. At 72 hours the most active regeneration is seen. In one instance in the 96 hour period there is marked evidence of endothelial and connective tissue proliferation with resulting scars presenting a picture suggesting early cirrhosis. The scars are intralobular in character. The other 96 hour sections show only slight remaining evidence of the injury. Except for one instance (Rat 611, 120 hour) showing focal areas of connective tissue and endothelial proliferation, all of the 120 and 144 hour sections show the normal liver with no evidence of scarring or other traces of the injury.

Protein Diet.—The extent of the necrosis is less than is observed on the other diets although a larger amount of chloroform was ad-

ministered. Two series were studied, with dosages of 1.5 and 2.5 cc. per kilo, respectively. The findings in the two series parallel one another. The reparative process is less active from a histological view-point than on the carbohydrate and standard diets. There is little or no leucocytic infiltration and mitoses are few and far between. In the 72 and 96 hour periods the nuclei of the hepatic cells vary greatly in size and chromatin content. In many areas two nuclei are seen within a single cell, suggesting direct cell division. In some instances after the 96 hour period accumulations of fat are seen in the central portions of the lobule. In the 120, 144, and 168 hour sections the repair is complete without resulting scars.

High Fat Diet.—The necrosis is more extensive than on the standard, the high carbohydrate, or high protein diets. There is extensive hemorrhage into the areas of necrosis. The infiltration with phagocytic cells is less obvious and the number of mitotic figures is definitely smaller than is seen on the standard diet. In the 72 hour period, some sections show slight connective tissue and endothelial proliferation in the central portions of the lobule. In others, wide areas of hyaline necrosis persist (Fig. 3). In the 96 and 120 hour sections the reparative process is less advanced than on the standard and carbohydrate diets. The sections, in these periods, show small central areas in which the regeneration of the liver cells is not complete and there is a varying amount of fat around the central vessels. In some cases the connective tissue and endothelial proliferation is very conspicuous (Fig. 4). This is also present in one 144 hour section.

In the 144 and 168 hour sections the liver regeneration is apparently complete although there is a considerable amount of fat in the central zones of some sections.

DISCUSSION.

Various theories have been advanced to account for the peculiar toxicity of chloroform. Opie (1914) suggested that the mutual solubility of fat and chloroform tended to concentrate its effect in the liver. Graham (1915) showed that a similar, though not identical, lesion was produced by intraportal injections of small amounts of hydrochloric acid and postulated that the poisonous effect of chloroform

was due to hydrochloric acid formed in its decomposition.[2] Neither Davis and Whipple (1919) nor Underhill and Kapsinow (1922), attacking the problem from different points of view, have been able to corroborate the suggestion of Graham. All who have worked on this problem recognize the increased toxicity on diets high in fat. Simonds (1919) states that the oxidative powers of the animal are low after chloroform poisoning. The well known difficulty of oxidation of fats in the body may account for this greater toxicity in such diets no matter what theory is invoked to explain the mechanisms of the initial tissue damage.

Our data indicate a relatively marked degree of resistance to the deleterious effects of chloroform in animals fed diets high in protein. This observation taken in connection with the smaller extent of necrosis observed in rats given the maximum non-lethal dose of chloroform seems to show that protein exerts a protective action on the hepatic cells in the presence of the poison or its decomposition products. What the mechanism of this protective action maybe, we are unable to say at present.

We have confirmed other workers in this field in the high toxicity in animals on high fat diet and in the somewhat greater resistance of those on high carbohydrate diet. A comparison of the diets on the basis of the resistance to the toxicity of chloroform conferred by them shows the following: protein > carbohydrate > standard > fat.

A comparative study of the repair of the liver on a standard balanced diet, a high carbohydrate diet, a high protein diet, and a high fat diet shows differences between the toxicity of chloroform and the rate and character of the reparative process under the conditions of these experiments.

The reparative process is most active on the standard balanced diet and there is a complete return of the liver to the normal anatomical picture without evidence of scarring. In the 96 hour sections there is very little remaining evidence of injury, and in the 120 and 144 hour sections the repair is complete.

[2] Schultz, Hall, and Baker (Schultz, E. W., Hall, E. M., and Baker, H. V., *J. Med. Research*, 1923, xliv, 207) have shown that periportal areas of necrosis are obtained when chloroform is injected into the portal vein of dogs. The lesions described by Graham were apparently similarly located.

On the high carbohydrate diet the necrosis is slightly less extensive. The reparative process, judging largely by the numbers of mitotic figures, is less active in the early periods than on the standard diet. In the 96 and 120 hour periods an interesting difference is observed. There is quite marked proliferation of the endothelium and the connective tissue stroma in the injured areas. This is so marked in some sections that a histological picture of cirrhosis of the liver is seen, although it is usually stated that the repair of a chloroform necrosis of the liver takes place without resulting scars or other evidences of the injury (Whipple and Sperry (1909)). A similar picture has been described by Pearce (1906) in the regeneration of necroses in the liver produced by the intravenous injection of hemagglutinative sera. In this case, however, the injury differs from that produced by chloroform in that the hepatic cells and endothelial cells are completely destroyed while in the chloroform injury only the parenchymatous cells are affected. These findings with the results in later periods will be described in detail in another communication.

On the high protein diet, in spite of much larger doses of chloroform, the necrosis is considerably less extensive. The actual time interval after which repair is complete may be slightly earlier than on the other diets, *i.e.*, at 96 hours, although the activity of the repair, as may be judged from the histological picture is less striking than is observed in the standard diet. The difference in the time of healing is very probably due to the less extensive injury and not to a dietary factor. There are no areas of scarring.

On the high fat diet the necrosis is more extensive than is observed on the other diets and the rate of repair is definitely less advanced at a given period. The cicatrization described above on high carbohydrate diet is even more extensive on the high fat food. These scars are observed in 144 hour sections (Rats 650 and 730) but in the remaining 144 and the 168 hour sections the regeneration is complete although a considerable amount of fat is present in the central zones of the lobules in some instances.

In regard to the cicatrization mentioned above it is interesting to note that this finding is most conspicuous on the high fat diet, which shows the slowest rate of repair and is completely absent on the standard diet in which the repair is most active. The scars are not seen in

the high protein diet animals. It is suggested that these scars are indicative of and possibly result from a delayed process of repair. They are not associated with the extent of the necrosis only, as they are observed in the high carbohydrate diet animals showing a less extensive necrosis than the standard diet animals in which scars were not found.

Our results on the slower rate of regeneration on the high fat diet in rats confirm those of Whipple and his collaborators on dogs. On the other hand, the most rapid rate of healing was observed in our experiments on the standard diet and not on the high carbohydrate diet as reported by them on dogs. In comparing the process of repair on the protein and carbohydrate diets we find that they differ chiefly in the absence of any connective tissue and endothelial proliferation on the protein diet. This may be indicative of a slower rate of repair on the carbohydrate diet. Finally, in comparing the activity of the process of repair, the standard diet is first and the high fat diet last, with little if any difference between the carbohydrate and protein diets.

CONCLUSIONS.

1. The toxicity of chloroform varies according to the diets used in these experiments in the following order of decreasing susceptibility of the animals: high fat > standard > high carbohydrate > high protein diets.

2. On the high fat and high carbohydrate diets there may be a more or less marked proliferation of the endothelium and the connective tissue stroma in the necrotic area producing in some instances scars resembling the picture of an early cirrhosis.

3. On the diets studied, standard, high carbohydrate, high protein, and high fat, the most active and rapid repair is observed on the standard balanced diet. On the high fat diet the reparative process is definitely delayed in comparison with the others. There are only slight differences between the high carbohydrate and high protein diets which suggest but do not conclusively show a more rapid repair with the latter diet.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Rat 429. Standard diet. Chloroform necrosis of liver 24 hours after injection. There is an extensive central necrosis. Only a narrow rim of uninjured liver cells remains at the periphery of each lobule. × 150.

FIG. 2. Rat 309. Standard diet. Chloroform necrosis of the liver 72 hours after injection. The reparative process is strikingly active. There are large numbers of mitotic figures. A large proportion of the injury has been repaired. × 150.

PLATE 2.

FIG. 3. Rat 668. Fat diet. Chloroform necrosis of the liver 72 hours after injection. A large area of hyaline necrosis persists. The reparative process is not active. Mitotic figures are inconspicuous. × 150.

FIG. 4. Rat 656. Fat diet. Chloroform necrosis of the liver 96 hours after injection. The picture resembles a cirrhosis of the liver. There is a striking proliferation of the connective tissue and endothelial cells within the injured area. The scars are intralobular. × 150.

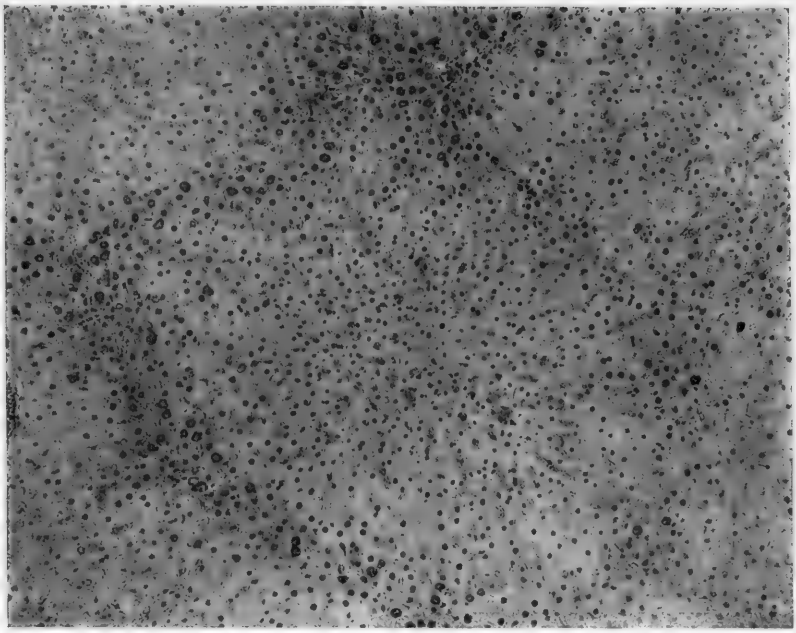

FIG. 1.

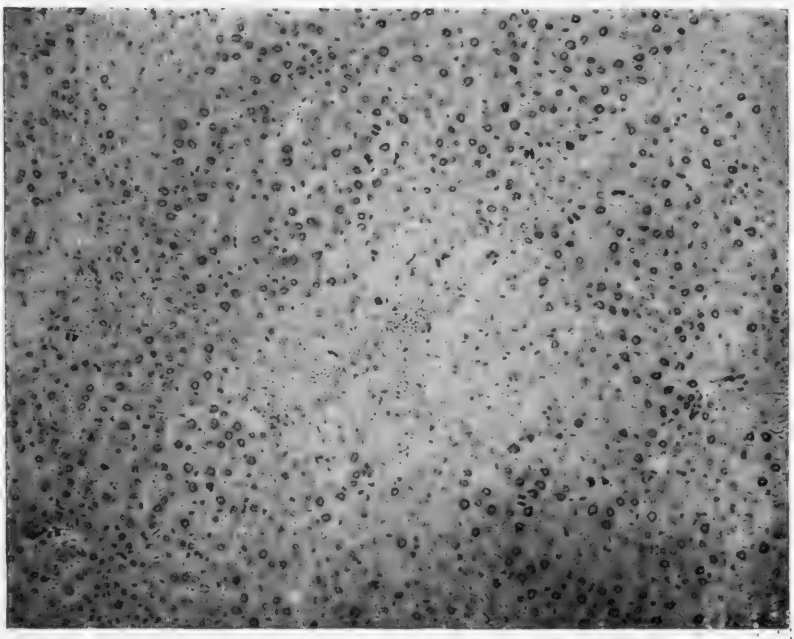

FIG. 2.

(Moise and Smith: Tissue regeneration. I.)

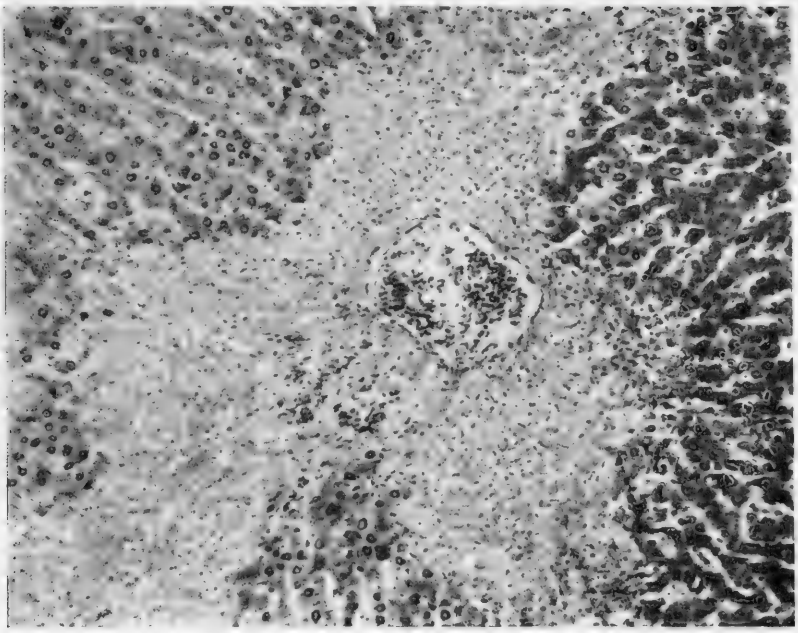

FIG. 3.

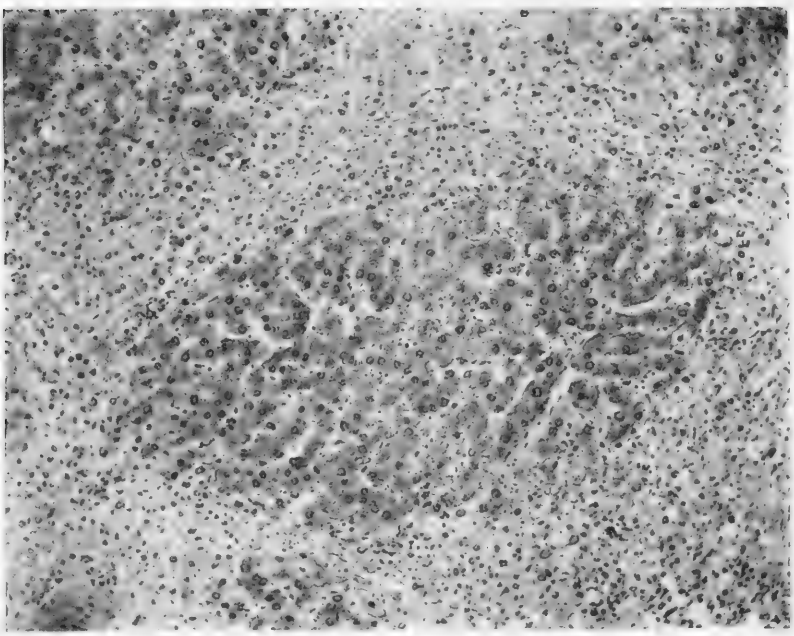

FIG. 4.

(Moise and Smith: Tissue regeneration. I.)

STUDIES ON THE TOTAL BILE.

VI. The Influence of Diet upon the Output of Cholesterol in the Bile.

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(Received for publication, March 29, 1924.)

INTRODUCTION.

In previous communications from this laboratory[1-3] certain immediate aspects of the problem of cholelithiasis have been taken up. In the present paper a more remote though not less important one will be considered, namely that of whether the output of cholesterol in bile can be influenced by diet. The matter is of importance not only theoretically but in connection with a possible prophylaxis for stones. The published data bearing upon it are indecisive for many reasons.

Often inadequate methods of bile collection have rendered void the results of careful chemical analyses while in the few instances in which all of the secretion has been obtained, the conditions have been such as to favor gall bladder inflammation, and cholangitis—conditions both which may lead to a local elaboration of cholesterol[4] into the bile. Analyses of the contents of the gall bladder and ducts at autopsy which some authors have relied upon cannot be expected to give trustworthy indications as concerns secretion during life. In experimental work on animals, the fistula of Schwann has been universally employed with the result that there are as yet on record no data concerning the cholesterol content of hepatic bile uninfluenced by the gall bladder—an organ which has been shown to modify the secretion markedly.[5]

Rous and I have recently developed[6] a method for the collection of the total bile of animals from day to day under sterile conditions and

[1] Rous, P., McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1924, xxxix, 77.
[2] Rous, P., Drury, D. R., and McMaster, P. D., *J. Exp. Med.*, 1924, xxxix, 97.
[3] Drury, D. R., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1924, xxxix, 403.
[4] Naunyn, B., Klinik der Cholelithiasis, Leipsic, 1892.
[5] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.
[6] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

over long periods of time. The secretion is not exposed to the gall bladder. The method has afforded opportunity for precise studies of the individual bile constituents.[7-11] The animals yielding the secretion remain in excellent condition. It is possible to determine accurately the total cholesterol output over a period of weeks or months.

Previous Literature.

Numerous authors have reported a rise in the cholesterol of blood after feeding the substance or giving diets rich in it.[12-15] Experimental obstruction of the bile duct of dogs results in hypercholesterolemia[16] and the condition has been met with in clinical cases of biliary obstruction.[17-19] Gardner and Fox[20] have shown a greater excretion of it than intake and suggest synthesis within the body; and Stepp,[21] among others, holds that a lipoid-free diet gives lessened biliary output of cholesterol. Chauffard[22] and Bacmeister and Havers[23] insist that there is a relationship between hypercholesterolemia and gall stone formation.

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[7] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[8] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.

[9] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 685.

[10] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.

[11] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 733.

[12] Grigaut, A., and l'Huillier, A., *Compt. rend. Soc. biol.*, 1912, lxxii, 304.

[13] Lemoine, G., and Gérard, E., *Bull. et mém. Soc. méd. Hôp. Paris*, 1912, xxxiii, series 3, 931.

[14] Widal, F., Weill, A., and Laudat, *Semaine méd.*, 1912, xxxii, 529.

[15] Henes, E., *Deutsch. Arch. klin. Med.*, 1913, cxi, 122.

[16] Chauffard, A., Laroche, G., and Grigaut, A., *Compt. rend. Soc. biol.*, 1913, lxxiv, 1093.

[17] Chauffard, A., Laroche, G., and Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxx, 20; *Ann. méd.*, 1920, viii, 149.

[18] Schmidt, H. B., *Arch. Int. Med.*, 1914, xiii, 121.

[19] Rothschild, M. A., and Rosenthal, N., *Am. J. Med. Sc.*, 1916, clii, 394.

[20] Gardner, J. A., and Fox, F. W., *Proc. Roy. Soc. London, Series B*, 1921, xcii, 358.

[21] Stepp, W., *Z. Biol.*, 1919, lxix, 514.

[22] Chauffard, A. M. E., Leçons sur la lithiase biliare, Paris, 1914.

[23] Bacmeister, A., and Havers, *Deutsch. med. Woch.*, 1914, xl, 385.

The works of Goodman,[24] Röhmann,[25] Aschoff,[26] and of Bacmeister[27] since 1907–08 have convinced them that the cholesterol of bile varies greatly with dietary changes and that, as found in the bile, it is a product of the general metabolism, not elaborated by the mucosa of the biliary passages as Naunyn,[4,28] Doyon and Dufourt,[29] and Kausch[30] believe. On the other hand, authors are not wanting who question these views on the basis of their own findings—Lichtwitz,[31] D'Amato[32]—and claim that the evidence sustaining it is faulty. Some of the reasons for doubting such evidence have already been given in the introduction to this paper. Stadelmann[33] has suggested an origin of the bile cholesterol from degenerating liver cells and duct epithelium; and Doyon and Dufourt[29] have demonstrated that the cholesterol content in human gall bladder bile can be much reduced by filtering out the cellular elements; and Naunyn, as already mentioned, has long championed the view that the substance is principally derived from the lining of the bile passages.

These various views and findings will suffice to illustrate the disputations and contradictions which abound in the literature.

Selection of the Method of Quantitation.

All the methods for quantitative determination of cholesterol in the bile leave something to be desired. They fall into two general groups, one involving an extraction of the substance and its precipitation as "digitonin cholesteride," as originally proposed by Windaus, the other extraction and colorimetric determination as first suggested by Grigaut[34] and later adapted by many authors, most usefully by Autenrieth and Funk.[35]

[24] Goodman, E. H., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 91.

[25] Röhmann, F., *Berl. klin. Woch.*, 1912, xlix, 1993.

[26] Aschoff, L., *Dermatologien Studien*, 1910, xxi, 23.

[27] Bacmeister, A., *Münch. med. Woch.*, 1908, lv, 211, 283, 339.

[28] Naunyn, B., *Münch. med. Woch.*, 1898, xlv, 1293.

[29] Doyon and Dufourt, *Arch. physiol. norm. et path.*, 1896, xxviii, series 5, 587.

[30] Kausch, W., Ueber den Gehalt der Leber und der Galle an Cholestearin unter pathologischen Verhältnissen, Inaugural dissertation, Strasbourg, 1891.

[31] Lichtwitz, L., Ueber die Bildung der Harn- und Gallensteine, Berlin, 1914.

[32] D'Amato, L., *Biochem. Z.*, 1915, lxix, 217.

[33] Stadelmann, E., Der Icterus und seine verschiedenen Formen. Nebst Beiträgen zu Physiologie und Pathologie der Gallen secretion, Stuttgart, 1891.

[34] Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxxi, 513.

[35] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 243.

As a purely chemical procedure, the method of Windaus is superior, but for practical purposes, when large numbers of determinations are to be made, the time consumed in precipitation and gravimetric procedures has to be considered, not to speak of that required for recovery of the substance. Other and more serious difficulties also present themselves. Pure digitonin is at present difficult to obtain and is, moreover, exceedingly costly. Again when working with dog bile, the precipitate of "digitonin cholesteride" is at times so small that the gravimetric error may be considerable. The disapproval by chemists of the Autenrieth-Funk method is centered about the color reaction, upon which quantitation is based. The green color employed in the readings changes rapidly, that is to say in the course of a few minutes, to yellow. Many authors take note of this difficulty. However, the figures of Klinkert,[36] Mueller,[37] and others show clearly that the method compares favorably with that of Windaus when carefully followed and is sufficiently dependable to yield useful results. Mueller[37] reports a comparison of the two methods and shows that if properly performed the colorimetric quantitation is as reliable as that by precipitation. Bloor and Knudson[38] state, concerning colorimetric methods: "The procedure in these methods is relatively simple, the time required short, and the color a satisfactory one to measure. They give, moreover, higher values than the gravimetric method."

In view of the objections to the digitonin method mentioned above, and owing to the large number of determinations to be made, it was deemed advisable to choose the colorimetric one—the more so since for the purpose of the work separate determinations of free and combined cholesterol were not desired.

Modifications.

With these considerations in mind, attempts were made to improve the quality of the green in the Autenrieth-Funk color reaction—the chief source of error in the method. The further change was adopted of substituting for the cholesterol standard solution an inorganic one with a slight element of yellow in the green, which more nearly approached the color obtained with bile samples, for a true color match greatly facilitates the colorimetric readings.

The yellowish tint appearing in the color reaction has been ascribed by some[38] to the action of the KOH used in extracting, but we have found it present after

[36] Klinkert, D., *Berl. klin. Woch.*, 1913, l, 820.

[37] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

[38] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, xxvii, 107.

the use of a hot alcohol and ether mixture as advocated by Bloor[39] and also on simple extraction with ether in a Soxhlet apparatus. Mueller[37] noticed it even in a reading made with pure cholesterol. The discoloration sometimes appears as the color change takes place in extracts previously colorless. The inclination is now to consider it due to a changed cholesterol or a cholesterol-like substance, perhaps an hydroxycholesterol[40] originally present in the bile. Evidence for this belief may be found in the fact that the bile of certain dogs used in the present work always gave a strongly yellowish color, that of others never. The bile of the worst offender was infected, that of the others ordinarily sterile. Mueller[37] gives a striking demonstration that some other substance or substances may add to the green in colorimetric determinations, and that they are closely related to cholesterol, perhaps hydroxycholesterol.

For the colorimetric test, as generally described in the literature, an aliquot portion of the ethereal extract is evaporated and taken up in chloroform. To this 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid are added and the mixture is made up to a fixed volume and read at room temperature some minutes afterwards. Care must be taken to use always the same quantity of acetic anhydride and acid.

A far richer green can be produced for comparison by using the entire extract from 2.5 or 5 cc. of dog bile rather than an aliquot portion of it. The stronger green is more easily read in the colorimeter. If, now, the reaction is brought about very rapidly, this pure green develops prior to the yellow discoloration and a much clearer reading is obtained. For the purpose I have used hot chloroform and hot acetic anhydride; and the mixing flask has been kept warm, so that the reaction takes place at 50° to 55°C. The rich green color appears almost at once and prior to the yellowish discoloration. Readings can be made before this develops and they can be repeated at frequent intervals during the next few minutes. The readings nearest the moment when the color best matches the standard solution are taken and averaged. In this way, far better color matchings are obtained, while repeated readings of a single bile are found to check closely.

All the bile specimens were compared with the inorganic standard to be described below. In this way any errors referable to color differences were rendered constant throughout the whole study.

The value of the inorganic standard solution was determined by repeatedly reading it against known cholesterol solutions in chloroform. The fact should again be emphasized that in carrying out determinations, exactly the same procedures were followed in every case as regards the time and the quantities of reagents used.

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[39] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[40] Lifschütz, J., *Münch. med. Woch.*, 1913, lx, 1549, 2346.

Method.

All estimations are made in duplicate and at times quadruplicate.

5 cc. of bile are mixed with 45 cc. of 20 per cent KOH, placed in a glass stopper bottle in the boiling water bath for 2 hours, cooled, and about 175 cc. of ether added to the bottle. This is shaken mechanically for ½ hour, the ether poured off and filtered, another 175 cc. added, and extraction repeated. The large amounts of ether and the prolonged shaking render further extraction unnecessary. The ether may be shaken with water to wash it and with 1 per cent sulfuric acid to remove any KOH present, though this was not always done. It is then filtered and the combined extracts evaporated to dryness. The dry residue is repeatedly extracted with reagent chloroform, the combined extracts evaporated to a value of less than 7 cc. and transferred to a 10 cc. volumetric flask and warmed to 55°C.

Color Reaction and Reading.—To the warm volumetric flask containing the extract 2 cc. of warmed acetic anhydride and 0.1 cc. concentrated H_2SO_4 are added and the volume made up to 10 cc. with hot chloroform. The whole is transferred at once to a Duboscq colorimeter cup and the readings begun as soon as possible. They are made every few seconds while the green color reaction develops, which happens very rapidly. After a few minutes, the hue becomes yellowish and no longer compares well with the standard solution. The ten readings taken when the color most closely resembles that of the standard solution are averaged and used for the quantitation.

Inorganic Standard Solution.—A stock solution of approximately 10 per cent copper sulfate in water (I used 100.005 gm. of copper sulfate crystals made up in water to a volume of 1,000 cc.) and acidified with 1 cc. concentrated H_2SO_4, and another stock solution of 1 per cent potassium bichromate are kept in readiness. For the readings of each day, 0.50 cc. of the bichromate solution is added to 10 cc. of the copper sulfate solution. The resulting color is a slightly yellowish green which matches that most generally given by the cholesterol. The solution as employed for the present work had the color strength of cholesterol in a concentration of 0.143 mg. per cc. This was ascertained by reading it against known cholesterol (Kahlbaum) solutions in chloroform.

These deviations from the ordinary colorimetric method of estimating cholesterol have been described at some length because they greatly increase its accuracy.

The intubated dogs employed for the work were for the first few days fed on a mixture of bread and milk, given as a routine. After the first 10 days of intubation they received in addition 450 gm. of lean meat mixed with this, and the quantity of food taken each day was recorded. On such a diet they remained in excellent condition throughout the period of the experiment. The bile was collected under

aseptic conditions at intervals of 24 hours for periods of weeks. Cultures were regularly made from it on agar and in bouillon; and from time to time portions were centrifuged and the sediment stained and examined for bacteria. The secretion proved sterile save in one instance, of Dog 18, Text-fig. 3, in which it became infected with a saprophyte at the 15th day of collection.

Cholesterol Output in the Postoperative Period.

The output of bile pigment, once the dog has recovered from the initial operation, is extremely constant under normal conditions[5,7] being uninfluenced by dietary changes. The cholesterol output, by contrast, tends to vary greatly and only approximates uniformity when the dietetic conditions remain the same from day to day. The bile secreted in the first 24 to 48 hours after operation is scanty and more highly pigmented than in the days next succeeding, when the fluid quantity gradually increases and that of the bile pigment drops off to reach a level after some 6 or 7 days. Even when great care has been taken to prevent extravasation of blood into the tissues at the operation, the pigment yield in the bile is higher for a day or so thereafter than at later periods. In the case of the animals of the present work, the bilirubin curves, following the postoperative 24 to 48 hours, underwent the usual slow drop to below the initial yield.

The changes in the cholesterol output were very different. The output of the 1st day after operation was small as compared with that of the 2nd or 3rd day. Similar changes have been noted too by Fasiani[41] in bile samples collected from time to time during each day. They may conceivably be the result, like the initial pigment changes, of some breaking down of blood. Later the cholesterol falls again but, unlike the bilirubin, the amount put out remains greater than on the 1st day. As an example of this Dog 16 put out 0.582 mg. of cholesterol per hour on the 1st day after operation. This rose to 2.05 mg. on the 3rd day, and between the 6th and 10th days had fallen to 1.27 mg. per hour. The specimens from seven other dogs showed alterations roughly similar to these.

[41] Fasiani, G. M., *Arch. ital. biol.*, 1915, lxii, 136.

The Relation between Bile Quantity and Cholesterol Output.

Throughout the later collecting period, abrupt day-to-day variations in the output of cholesterol were frequent as will be evident from inspection of Text-figs. 1 and 7. The influence of food differences upon the yield was found to be a direct and important one. The character of the diets has been indicated on all the charts and the amount of food eaten each day recorded. As "ordinary" diet, the animals were given bread and milk, and as "mixed" diet, 450 gm. of lean, raw meat, chopped fine and kneaded up with 1,050 gm. of bread wet with milk. Where special diets such as liver or cholesterol-rich foods were given special symbols have been used to indicate the fact.

When the yield of cholesterol is compared with the amount of food eaten (Text-figs. 1 to 7), a direct relationship between the two is clearly to be seen. This relationship, however, is far from quantitative, but it supports the generalization that an animal eating well puts out more cholesterol in the bile than one eating poorly or fasting. Take the case of Dog 16, Text-fig. 1. The animal was in excellent health. On the days when it had eaten large amounts of food, cholesterol was secreted in relatively great quantity, (Specimens 18 to 25). At the same time the bile volume per hour was also in accordance with the well known fact that an animal eating well tends to put out more bile. An inspection of Text-fig. 1 clearly shows that the two are not essentially related. Often the cholesterol increase is out of proportion to the quantity of bile put out and rarely, too, an increase occurs with a decrease in bile volume. While the charts show occasional instances in which an increased ingestion of food failed to bring about an increase in the bile but was followed by a rise in the cholesterol output, many more examples are to be found in which it is accompanied by this cholagogue action. Instances of the sort first mentioned appear in Text-fig. 1, Specimens 20 and 21, Text-fig. 7, Specimens 6 and 7; and some of the second sort in Text-fig. 7, Specimens 8 and 10—following a day of fasting—and Specimens 14 to 17 after 3 days of fasting.

In connection with the effects of cholesterol-rich diets, some striking illustrations were found of the fact that the cholesterol yield is essentially independent of the bile volume. An instance of this appears

in Text-fig. 1, Specimens 57 to 59. On the 57th day after prolonged feedings with bone mash, the bile volume was high. On the 59th day, the first of fasting, the bile volume fell to less than half its figure, but the cholesterol output remained high with result in a great and almost reciprocal increase in its concentration. Again in Text-fig. 2, Specimens 24 and 25, obtained during cholesterol-rich feedings, and Text-fig. 6, Specimens 40 and 41, during a fasting period, drops in bile volume are seen without corresponding changes in the cholesterol secretion. In the animal last mentioned a drop in both did appear on the 42nd and 43rd days. Another variation is often observed, that of an increase in bile volume accompanied by a decrease in cholesterol. This can be found on Text-fig. 1, Specimens 34 and 35, 48 and 49, also in Text-fig. 4, Specimens 34 and 35.

Naunyn[4] holds that the cholesterol content of bile is independent of the diet and of alteration in the general metabolism. Jankau[42] fed dogs and rabbits 0.5 to 5.5 gm. of cholesterol and found no increase in the bile. Bacmeister and Havers[23] observed after food an increased yield in samples of dog bile but not in those from rabbits. Thomas's[43] findings indicate that the cholesterol content of dog bile is independent of food, but Goodman[24] points out that his work was done with samples of bile that represent the yield of but a few hours of each day only. This latter author reported that feedings of red corpuscles and rich protein diet greatly increase the cholesterol in dog bile. He, almost alone among workers, employed 24 hour specimens, obtained, however, from but one dog and under conditions which favored infection of the biliary tract. Lichtwitz,[31] in a discussion of Goodman's results, points out that the increase in cholesterol secretion after protein feeding was accompanied by an increase in the bile amount and he suggests that the increased bile flow merely carried out with it the cholesterol already present in the liver cells, hence the increased amount. Lichtwitz further comments that Goodman's results do not always point to a dependence of cholesterol output on the diet.

From the results here reported it may be concluded that increases in the bile amount and cholesterol output are usually associated, but this is far from being always the case. The cholesterol yield, then, is not essentially conditioned by changes in the bile volume though in general varying as this latter varies.

[42] Jankau, L., *Arch. exp. Path. u. Pharmakol.*, 1892, xxix, 237.

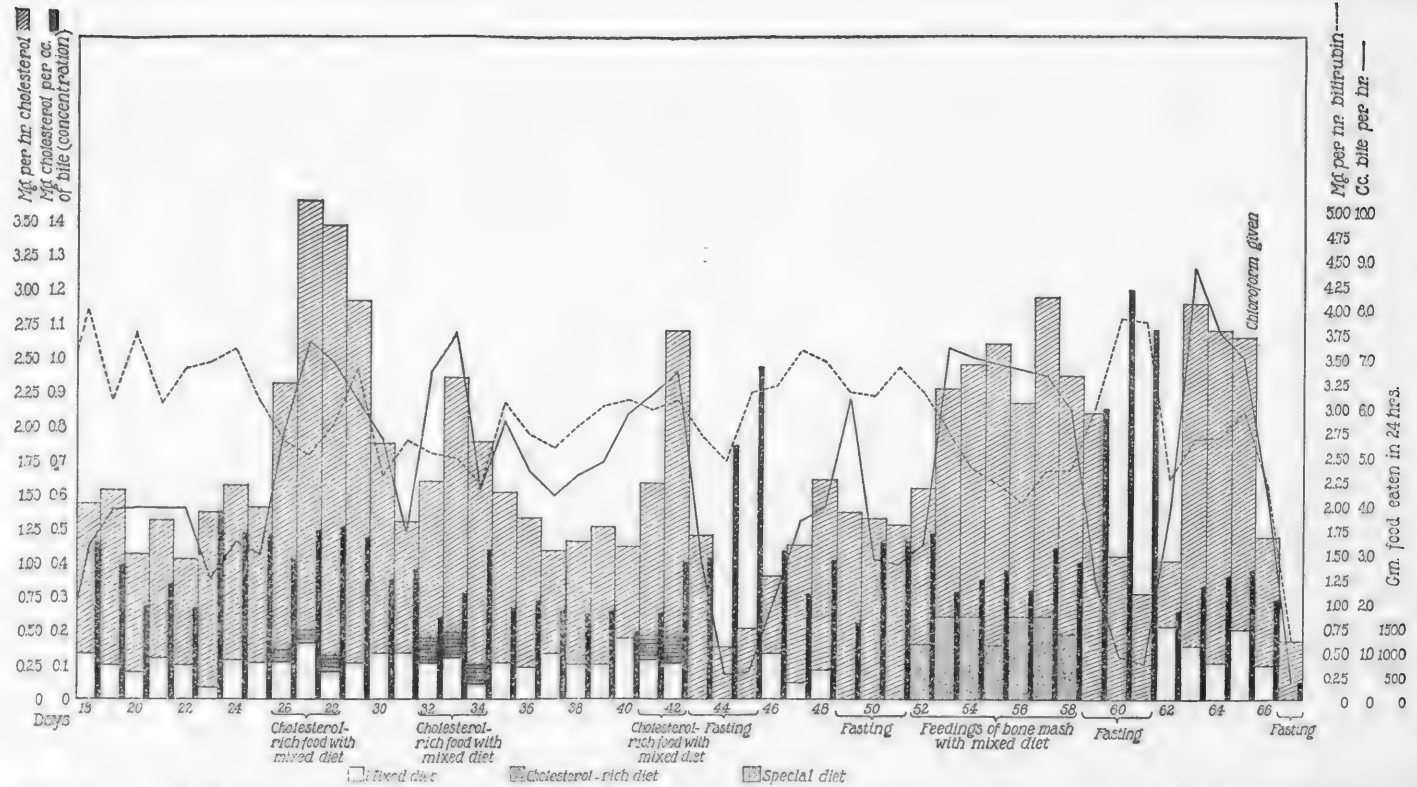
[43] Thomas, Inaugural dissertation, 1890. Cited by Goodman,[24] and by Lichtwitz.[31]

Fasting.

In contrast to the profuse thin, lightly pigmented, bile of a freely eating animal, the fasting dog puts out a scanty bile, thick and viscid. The amount secreted sometimes drops as in Dog 16, Text-fig. 1, from 159 to 11.5 cc. per day in 2 days time (Specimens 42 to 44), but without pronounced influence on the daily yield of bile pigment which is maintained through a reciprocal increase in concentration.[7] That the output of cholesterol is, by contrast, greatly disturbed can be seen from this chart and from others to be mentioned below. A dog eating poorly puts out a bile more concentrated in cholesterol, to be sure, but one containing far less of the substance in the total 24 hour specimen.

Text-fig. 5, 6, and 7, each embodying data from three different animals, illustrate the same striking phenomenon. In Text-fig. 5, during 3 days fasting, the cholesterol output fell from 2.0 to 0.54 mg. per hour (Specimens 12 to 15). At the same time the drop in the amount of bile secreted was from 8.3 to 0.5 cc. per hour. The concentration of cholesterol increased from about 0.57 to 1.12 mg. per cc., but this greater concentration was not sufficient to keep the 24 hour output of cholesterol at its usual level. The findings of Text-fig. 7 (Specimens 10 to 14) show almost the same thing. Text-fig. 6 from yet another animal shows a decrease in cholesterol output but no increase in cholesterol concentration with starvation (Specimens 41 to 43).

When food is given again after fasting, Dog 16 (Text-fig. 1), Specimens 43 to 46, 58 to 62, there occurs with the increase in the bile one in cholesterol output but not a proportionate one. The concentration of the lipoid in such biles is much lower than in fasting specimens, though high enough to yield, as just stated, a greatly increased amount during the 24 hours. It is not at all unlikely that this may be in part at least a consequence of a flushing out of the substance from the liver cells. In Dog 16 (Specimens 50 to 53) a decrease in cholesterol concentration did not occur with the resumption of feeding, but as it happened this animal was given a diet with bone mash containing some cholesterol.



TEXT-FIG. 1. Findings in an 18 kilo dog from the 18th to the 67th day after operation. There are obvious alterations in the cholesterol output associated with alterations in the type and amount of food eaten and changes also in the concentration of cholesterol and the bile volume. That the output of bilirubin and cholesterol vary independently is plainly shown (Dog 16).

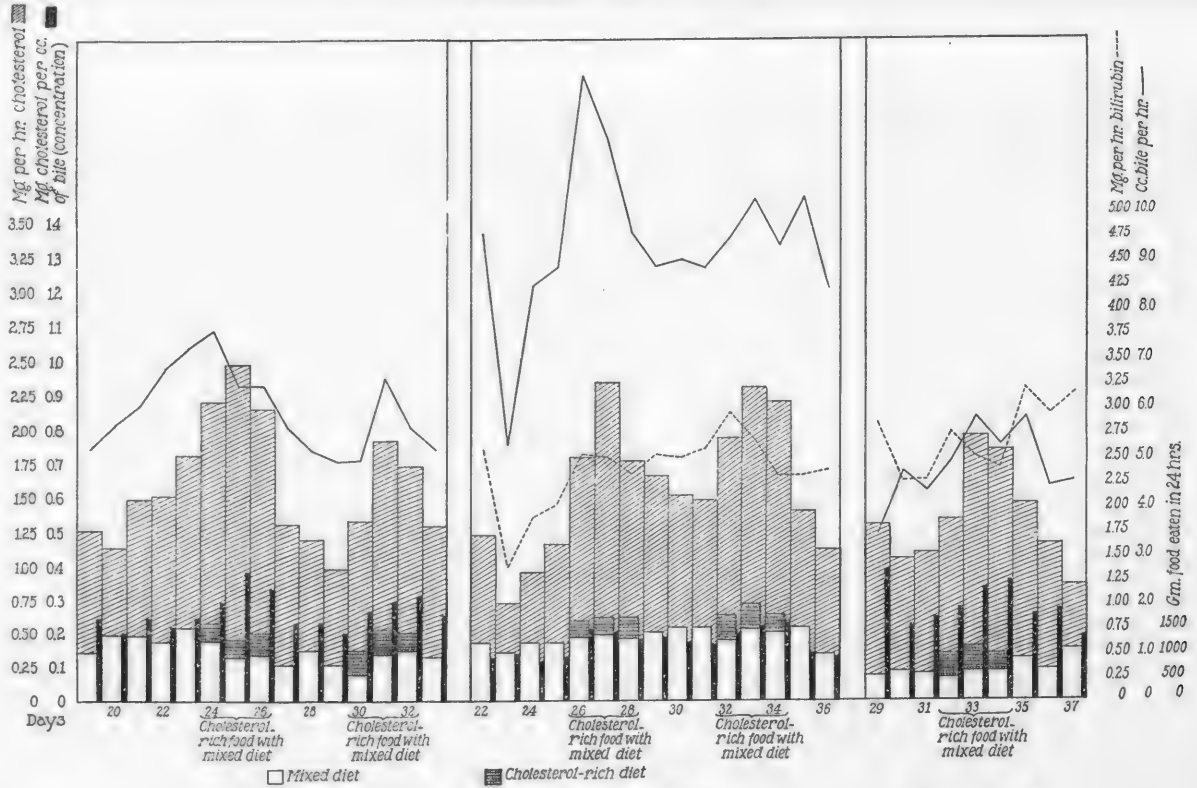
The Influence of Cholesterol-Rich Diet.

The protocols of most interest and importance were those dealing with the effect produced upon the cholesterol output by the administration of food rich in that substance.

The bile of four intubated dogs was followed for 25 days after operation, during which time they remained in good condition. The daily pigment and cholesterol output of each animal was determined. The pigment output after the postoperative period remained practically constant. The animals were now given 200 gm. of raw ox brains and three raw eggs daily for 3 days, in addition to the regular mixed diet. The eggs and brain were regularly eaten with relish before the routine food was attacked. Great increases in cholesterol output followed the giving of this material rich in it (Text-figs 1, 2, 3, and 4). A specimen protocol follows.

Dog 16.—Text-fig. 1. Operation as usual; ether anesthesia, dog in excellent condition after operation; on a mixed diet. The average amount of cholesterol in the bile was about 1.28 mg. per hour at the 18th day after operation. The animal was then eating 700 to 800 gm. of mixed diet, that is to say, bread, milk, and lean meat. On the 26th day after operation, 200 gm. ox brains and three eggs given, and the cholesterol output rose on the following day to 2.28 mg. per hour. On the 27th and 28th days this diet was continued, the animal eating in addition 500 to 1,000 gm. of mixed diet. The cholesterol output in the bile rose to 3.65 and 3.5 mg. per hour on these days, respectively. Mixed diet alone was given the following day, and the cholesterol at once declined to 2.94 mg. and on the next day to 1.86 mg. per hour. 3 days later cholesterol-rich food was again given and another, but less pronounced, rise occurred. The subsequent variations in output will be discussed further on.

Text-figs. 2, 3, and 4 illustrate the changes that took place in the other dogs. Every time cholesterol-rich food was given, the cholesterol output in the bile increased pronouncedly. Not only was a greater amount present but the concentration of cholesterol per cc. of bile was also greater. This is a fact of much interest in connection with the relationship already discussed between bile volume and cholesterol yield. The combination of increased volume with increased concentration yielded an amount of cholesterol totally out of proportion to the change in the rate of bile secretion. In cases of the sort the greater output of cholesterol sometimes appeared at once,


Mg. per hr. cholesterol
Mg. cholesterol per cc. of bile (concentration)
Mg per hr. bilirubin
Cc. bile per hr.
Gm. food eaten in 24 hrs.
Days
Cholesterol-rich food with mixed diet
Cholesterol-rich food with mixed diet
Cholesterol-rich food with mixed diet
Cholesterol-rich food with mixed diet
Cholesterol-rich food with mixed diet
Mixed diet
Cholesterol-rich diet

TEXT-FIG. 2. TEXT-FIG. 3. TEXT-FIG. 4.

TEXT-FIGS. 2, 3, and 4. Changes in the cholesterol output in bile after and during feedings rich in cholesterol to three dogs. Each time cholesterol-rich food was given a great increase in the cholesterol of the bile occurred, and usually a greater concentration of the substance as well (Dogs 26, 18, 20).

that is to say, in the bile secreted on the same day the food was given, while again it appeared on the day after. Similarly the abundant output may continue for 24 hours after high cholesterol foods have been withdrawn from the diet.

Dog 16.—Text-fig. 1. Specimens 27 and 28, previously mentioned. The output on the 2nd and 3rd days of lipoid feeding yielded more than twice the usual amount of cholesterol, and on the day following withdrawal of this food almost as much was obtained though the animal received only a mixed diet. Thereafter the cholesterol fell to the ordinary level. Later, the same dog, fasted for a day, after a feeding with bone mash diet containing cholesterol, secreted during this fast period an unusually large amount of cholesterol. Text-fig. 1, Specimens 58 and 59.

Dog. 26.—Text-fig. 2. Specimen 24. Exhibited on the same day on which it was fed cholesterol-rich diet, an increase of the substance in the bile, though a more moderate one. There was a return to the ordinary output almost at once after withdrawal of the lipoid-rich diet. In this animal the cholesterol rose from 1.50 mg. per hour to 2.46 mg. on the 2nd day of feeding, nearly double the average yield.

Dog 18.—Text-fig. 3. Specimens 26 to 28. An increase in cholesterol of the bile to more than double the average amount occurred after feeding with ox brains and eggs, that is to say, it rose from 1.14 to 2.33 mg. per hour.

Dog 20.—Text-fig. 4. Specimens 32 to 34, previously mentioned. Again an almost similar rise occurred, from 1.07 to 1.93 mg. per hour. Although the response of both these dogs was prompt and occurred on the same day as the cholesterol feedings, the effect lasted for a brief period after the feedings ceased. This was the usual case.

An unexpected finding which deserves comment presented itself in the course of a later experiment for another purpose. During a study of the calcium content of the bile, Dog 16 was fed a bone mash mixture (200 gm.) daily in addition to the usual diet, Text-fig. 1, bile Specimens 52 and 58. The bile volume increased and the cholesterol output underwent a rise from 1.25 mg. per hour on the day before the feedings were commenced (Specimen 51) to about 2.5 mg. and even more during the following 6 or 7 days. An analysis of the bone mash mixture proved it to contain but 0.1 per cent of cholesterol. Since the animal ate 200 gm. of it, in addition to the regular food, this increase of but 200 mg. of cholesterol over the previous average in the diet was followed by an increased output of 25 mg. per day in the bile. The increase in the substance was almost as great as that which

followed the addition of cholesterol-rich foods to the diet in the experiments just described.

Some authors have raised the objection that even should the cholesterol of bile be increased by ox brain feedings this must be considered as the result of highly abnormal circumstances, since the animal is so to speak overloaded with cholesterol thereby.[44] The results with bone mash would suggest that such a result can be obtained with substances containing cholesterol in relatively small amount, when taken in addition to the ordinary ration. The possibility that bone mash as such, independent of its cholesterol content, has some influence on cholesterol metabolism, which is not probable, must also be considered.

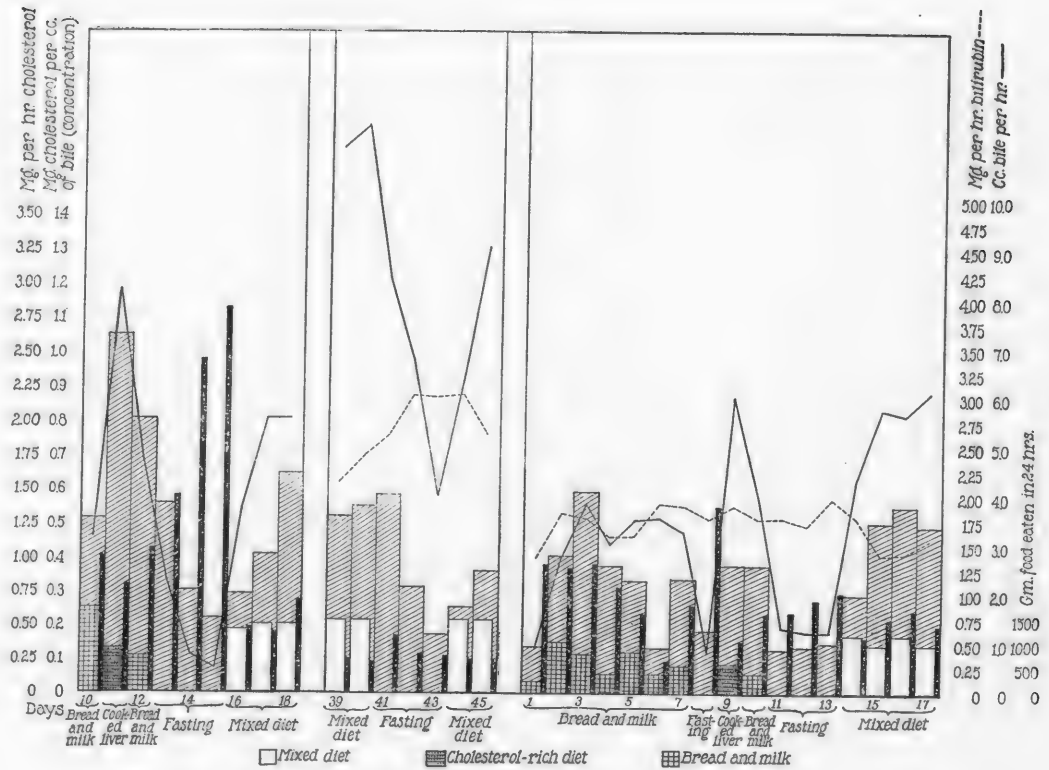
Concentration.

The cholesterol concentration of bile obviously does not bear a reciprocal relation to its amount. What factors influence it?

That concentration is independent of any one factor will be apparent on reference to the charts. When a cholesterol-rich diet is given, a slight rise in the concentration of cholesterol usually accompanies the increase in the volume of bile. (Text-fig. 1, Specimens 26 to 28, 32 to 34, 41 and 42, 52 to 58, and 62 to 65; Text-fig. 2, Specimens 23 to 25; Text-fig. 3, Specimens 25 and 26, 31 to 34; Text-fig. 4, Specimens 31 to 34.) At times, however, the concentration remains constant though the volume increases (Text-fig. 2, Specimens 20 to 23; Text-fig. 3, Specimens 26 to 28; and Text-fig. 5, Specimens 16 and 17). Text-fig. 5 shows the independence of concentration and volume for in Specimens 16 and 17 the concentration remained constant, whereas the bile output, and hence the cholesterol, increased, while in Specimen 18 the volume remained constant but the concentration and, as corollary, the total output of cholesterol increased.

By contrast, as already stated, a great rise in the concentration of cholesterol usually occurs with the decrease in the bile volume of the fasting animal (Text-figs. 5, 6, and 7, and in Text-fig. 1, Specimens 42 to 44, and 58 and 59). Finally, as in Text-fig. 1, Specimen 23, an increase in the cholesterol concentration and even in the total choles-

[44] Whipple, G. H., *Physiol. Rev.*, 1923, ii, 440.



TEXT-FIG. 5. TEXT-FIG. 6. TEXT-FIG. 7.

TEXT-FIGS. 5, 6, and 7. Effects of fasting in three different animals. A great decrease in the cholesterol output of the bile occurs, the concentration increases but not sufficiently to keep up the total amount. Here, as in the other charts the difference in the behavior of bilirubin and cholesterol is obvious.

terol as well may rarely accompany a decrease in bile volume. A study of these changes and of those occurring in Specimens 37 to 50, during which period the dog was given ordinary food, then ox brains, then fasted, and again given mixed diet and fasted yet again, impresses one with the essential independence of the cholesterol concentration and bile volume.

The completely different behavior of bilirubin in all these cases is of especial interest (see Text-fig. 7, for example).

DISCUSSION.

It is clear from the evidence here presented that the cholesterol quantity and concentration in dog bile are subject to profound modification by dietary influences. The changes are not slight and inconstant as the literature would lead one to expect.

The findings have an obvious, and not impossibly a considerable significance in relation to cholelithiasis. In this connection the facts should be recalled, however, that no cholesterol is found in gall stones of the dog[1,2]—doubtless because dog bile is notably poor in the substance and that the substance exists in the bile as an ester,[43] not as free cholesterol, as in human bile. But this does lessen the suggestiveness of the findings in connection with the problem of human stones. Were the amount and concentration of the cholesterol of the bile the sole factors in stone formation, it might well be that by a dietary regimen the likelihood of cholelithiasis could be much diminished. But unfortunately for generalization it is the state rather than the quantity of cholesterol which determines its deposition. Bile is highly complex and the equilibrium of cholesterol is determined in solution or suspension by certain other constituents which may vary with differing conditions. Nevertheless it seems reasonable to suppose that stone formation will take place more readily from a bile rich in cholesterol than from one poor in it.

There has been much said in the past of cholesterol elaboration by the gall bladder and duct walls.[28,4] In the present experiments the influence of the gall bladder was ruled out by the operative exclusion; and there was no inflammation of the bile passages or infection of the bile, save in the case of Dog 18, Text-fig. 3. The bile passages of this animal proved normal at autopsy, though the bile was infected in pure culture, evidently with a saprophyte.

SUMMARY.

The development of a method for the collection of total bile from dogs in a sterile state and uninfluenced by the gall bladder, day after day for weeks, has rendered possible an accurate study of the influence of diet upon the cholesterol output of the secretion.

When a diet rich in cholesterol is given the amount of the substance in the bile greatly increases. Not only this but the concentration per cc. is, in almost every instance, greater. An increase in the total food intake, by the addition to the ordinary ration of a bone mash diet containing only a slight additional amount of cholesterol (200 mg.) produces a similar, though lesser, increase. In the fasting dog, the cholesterol yield is greatly cut down.

The increase in the cholesterol after food rich in the substance does not depend on the cholagogue action of this latter, though it is true that the concentration of cholesterol in the bile usually increases with the bile volume.

Though fasting cuts down the cholesterol of bile, the concentration of the substance per cc. is greatly increased. On an ordinary diet the yield of it fluctuates abruptly and considerably from day to day. In general the rule holds that an animal eating largely puts out not only much more bile but much more cholesterol. The relation between bile quantity and cholesterol yield is anything but a fixed one, however.

The cholesterol yield of the bile does not parallel that of bilirubin. The pigment output from day to day remains relatively constant as compared with that of cholesterol.

FLAGELLAR AGGLUTININS.

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The question of the existence of flagellar antigen and agglutinins has been referred to in the literature at various times.

In 1903, Smith and Reagh[1] described flagellar and somatic agglutinins. They reviewed the papers published up to that time dealing with the part played by the flagella in agglutination reactions. By a study of the agglutinins produced by a motile and a non-motile strain of hog-cholera bacilli and by the examination of the two types of clumping occurring in these two sera they demonstrated the existence of the two agglutinins which they called somatic and flagellar agglutinins. The next year, Beyer and Reagh[2] studied the further differentiation of flagellar and somatic agglutinins by means of heat. They found that a temperature of 70°C. injured the flagella so that the motile bacilli were not agglutinated, but this temperature did not destroy their power of generating flagellar agglutinins in the animal body. A little later Smith and TenBroeck,[3] in an agglutination study with fowl and human typhoid cultures, observed evidence for flagellar and somatic agglutinins and explained certain reactions on the basis of their existence.

Recently (1922) Gruschka[4] reported variants occurring among Gaertner bacilli which showed differences in serological behavior. He studied the variants by receptor analysis and considered that the large clumping agglutinins were produced by labile H receptors and the fine clumping agglutinins by stable O receptors. Typically the Gaertner bacilli possessed both receptors. He found certain variants possessing O receptors alone, and others possessing H receptors with atypical O receptors. In his cultural study he observed differences in motility between the variants. Those forming large clumps, or possessing H receptors, were motile, and those forming only fine clumps, or possessing O receptors alone, were non-motile. He stated that his labile H receptor is probably identical with flagellar antigen.

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[1] Smith, T., and Reagh, A. L., *J. Med. Research*, 1903–04, x, 89.

[2] Beyer, H. G., and Reagh, A. L., *J. Med. Research*, 1904, xii, 313.

[3] Smith, T., and TenBroeck, C., *J. Med. Research*, 1914–15, xxxi, 503.

[4] Gruschka, T., *Z. Immunitätsforsch., Orig.*, 1922–23, xxxv, 97.

In this paper two groups of experiments are described, one following along the lines of the work of Smith and Reagh, but differing in that the motile and non-motile strains used were two types derived from a single strain originally motile and emphasizing therefore the factor of change. The second group of experiments deals with the separation of the flagella from the bodies of the bacilli and the direct preparation of a serum containing only flagellar agglutinins.

EXPERIMENTS.

Group 1. Experiments Dealing with the Change in Motility Occurring within One Strain and the Accompanying Change in Serological Behavior.

The hog-cholera culture Maryland was used in this work. Originally this culture was actively motile. It was used by Smith and Reagh in their experiments as a motile strain. This culture has been kept in stock on agar slants. In the spring of 1923, during some agglutination work when different colonies were picked and agglutinated, different reactions were noted with the separate colonies. When the cultures from these colonies were examined differences in motility were observed. The bouillon cultures of the colonies were replated and colonies again examined. The appearance of the colonies on the two plates was the same, but the hanging-drop examination continued to show the differences in motility. Agar slants were inoculated from individual colonies and these have been kept as stock cultures, being renewed every 1 to 2 months. At the present time the stock cultures of the two types have kept their different characters in respect to motility. Stained with a flagellar stain (Shunk[5]) the motile organisms showed numerous flagella, but in the preparation from the non-motile culture no flagella were seen. Culturally the two types are the same, at least in colony formation, growth in bouillon, and production of acid and gas from dextrose.

Serologically the two types differ. In an ordinary hog-cholera serum they gave the different types of clumping described for somatic and flagellar agglutination. In the production of immune serum Md.-motile produced a serum which agglutinated both types but with the

[5] Shunk, I. V., *J. Bact.*, 1920, v, 181.

expected differences in type of clumps and titer limit. It agglutinated Md.-motile 1:20,480, with soft, loose clumps, and Md.-non-motile 1:640, with small, fine clumps. Md.-non-motile produced a serum that agglutinated both types similarly to the titer 1:1,280. The clumps of the non-motile type were very compact, but those of the motile type were looser and more like the ones formed in the serum of the motile bacilli. However, a hanging-drop preparation of the clumps formed by the motile bacilli in their own serum, examined microscopically under the high power, showed no movement of the clumps, while such an examination of the clumps formed by the motile bacilli in the non-motile culture serum showed activity among the small clumps and a swaying motion throughout the larger clumps. This indicated that the motile culture serum contained an antibody acting directly on the flagella, but the non-motile culture serum was lacking in this antibody. In the absorption tests with the two types, the Md.-motile absorbed all agglutinins for both cultures from both sera, and Md.-non-motile absorbed only agglutinins for itself from the motile culture serum but all agglutinins from its own serum.

All these tests have shown that the only differences between the two types are the loss of flagella in one type and its different serological behavior. The agglutination results agree with the earlier reports for motile and non-motile strains. The fact that here the motile and non-motile types were derived from one strain which was originally motile but has changed, giving rise to a non-motile type, gave definite evidence that the change in motility or the loss of flagella affected the serological behavior both in the agglutination of the bacilli and in the production of agglutinins and therefore proved that the flagella are an antigenic factor giving rise to specific agglutinins.

Group 2. Experiments Dealing with the Separation of the Flagella from the Bodies of the Bacilli and the Direct Production of a Flagellar Antiserum Which Agglutinates Exclusively the Motile Bacilli.

Hog-cholera strain Md.-motile was again used for this work.

Preparation of Flagellar Suspension.—A Blake bottle of agar was inoculated with Md.-motile culture and incubated at 37°C. for 24 to

48 hours. At the end of this time a hanging-drop examination showed active motility, and a flagellar stain showed many flagella, some already detached from the bacilli. The growth was suspended in 10 cc. of 0.85 per cent NaCl solution and put into small bottles with a few glass beads. The suspension was shaken in a mechanical shaker for ½ hour and then centrifuged 1 to 2 hours. The shaking broke off the flagella and the centrifuging threw down the bulk of the bacilli but left the flagella in suspension. The supernatant fluid was drawn off with a pipette and shaken a second time to free the remaining organisms from the flagella. This suspension was centrifuged 4 to 6 hours. The fluid appeared perfectly clear. The upper half of the fluid was drawn off for use. Bacilli were not detected in a stained preparation, but a flagellar stain showed the presence of masses of flagella. This material was mixed with an equal volume of bouillon, and 0.5 cc. plated on agar to test for the presence of bacilli. Such plates sometimes were sterile, but sometimes it was necessary to centrifuge longer. After determining the probable sterility by plating, the whole suspension was heated ½ hour in a water bath at 60–62°C. and then incubated 24 to 48 hours to make sure of sterility.

Preparation of Flagellar Antiserum.—A rabbit was given three intraperitoneal injections of the flagellar suspension prepared as described above. The doses were 1, 1.5, and 2 cc., given at 5 day intervals. Each lot of material used for the injections was sterile and free from bacilli as far as determined by plating on agar before heating. The rabbit was bled 9 days after the last injection.

Agglutination tests were made with the serum obtained from the rabbit injected with the flagellar suspension. The results showed that the motile bacilli were strongly agglutinated, and the titer limit was 1:5,120, while the non-motile bacilli were not affected at all by this serum. This is very definite evidence for flagellar antigen since the flagella alone produced a serum which reacted exclusively with the motile type of bacilli.

The results of the agglutination tests of the Md.-motile and non-motile types with the three kinds of sera have been tabulated. These results clearly bring out the antigenic property of the flagella.

Absorption of the flagellar antiserum with the non-motile type of bacilli did not affect its agglutinating value. This showed that the

TABLE I.

Results of Agglutination Tests.

Type of bacilli.	Serum dilutions.										Salt solution plus culture control.
	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	
Flagellar plus somatic antiserum (whole Md.-motile culture).											
Md.-motile	C.	C.	C.	C.	C.	C.	++++	+++	+++	++	−
Md.-non-motile	"	"	"	++++	++	−	−	−			−
Somatic antiserum (Md.-non-motile culture).											
Md.-motile	C.	C.	C.	C.	++++	++	−	−	−		−
Md.-non-motile	"	"	"	"	++++	++	+	−	−		−
Flagellar antiserum (Md. flagella).											
Md.-motile	C.	++++	++++	++++	+++	+++	++	+	−	−	−
Md.-non-motile	−	−	−	−	−	−	−				−

bodies of the bacilli played no part in producing this antiserum, but the flagella alone gave rise to the antibodies present in the serum.

A direct flagella agglutination test was made by mixing the flagellar serum with the flagellar suspension. The result was a soft, delicate, rather fluffy precipitate visible in the first test in a dilution up to 1:80 and in a second test in a dilution of 1:320. This test was controlled first by using normal serum with the flagellar suspension instead of the flagellar serum, and secondly by mixing the flagellar suspension with salt solution instead of serum. Both sets of controls gave no precipitate. That the reaction was an agglutination rather than a precipitation was proved by carrying out a precipitation test using as antigen a filtrate of some of the same flagellar suspension used in the agglutination test. The result of the precipitation test was negative, showing that precipitins were not responsible for the reaction occurring between the flagellar serum and the flagellar suspension, but that the precipitate was the result of an agglutination or a clumping of the flagella into masses large enough to be seen. A preparation of the clumps examined microscopically in the dark field under the oil immersion showed the flagella distinctly. There were dense masses and on the edges a fine network and separate, single, wavy flagella were visible. Also a preparation stained with a flagella stain showed the single, wavy flagella tangled together into masses.

In the experiments described here one strain under three different conditions has been used to produce antibodies; namely, (1) the whole culture of the original motile type, (2) the non-motile type derived from it, and (3) the separated flagella. Each form has given rise to a serum containing either different antibodies or a combination of the two kinds. By means of the production of the three kinds of sera and the agglutination reactions of the three types of culture in these sera, the earlier work has been confirmed and more evidence has been furnished which proves the existence of the flagella as a separate antigenic factor giving rise to a specific agglutinin.

Another fact concerning flagella is their inability to take up the ordinary stains. They need a special treatment to prepare them for the stain. This indicates a different composition from the rest of the bacillus which is in agreement with the existence of their separate antigenic property.

CONCLUSIONS.

1. Certain bacilli within one strain may lose their flagella and become non-motile.

2. The change in serological behavior associated with the change in motility indicates that the flagella possess an individual antigenic property.

3. Flagella can be separated from the bodies of the bacilli.

4. A strictly flagellar antiserum can be produced by the injection of a flagellar suspension.

5. This direct production of a strictly flagellar antiserum is proof of the existence in flagella of a separate specific antigen.

THE COEXISTENCE OF PROTOZOAN-LIKE PARASITES AND MENINGOENCEPHALITIS IN MICE.

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PLATES 3 AND 4.

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The use of rabbits for the experimental study of the virus of encephalitis lethargica and allied conditions has been instrumental in directing attention to the existence of peculiar brain lesions first noted in these animals by Bull[1] and later by Oliver[2] who indicated the importance of securing definite information regarding their etiology. These lesions are disturbing factors for the reason that, although no signs of disease can be detected clinically, they are nevertheless to be found in about 60 per cent of ordinary stock rabbits considered as normal (McCartney[3]). Protozoan-like parasites have been observed to be associated with them by Wright and Craighead[4] and other investigators.[5,6]

The observations that we wish to describe briefly in this paper relate to the existence of similar lesions and parasites in apparently healthy laboratory mice.[7] In making our comparisons we have been able to supplement published accounts having reference to the rabbit with actual preparations which we have compared directly with the mouse material, lesion by lesion and parasite by parasite. For this privilege we wish to thank Dr. E. W. Goodpasture for his

[1] Bull, C. G., *J. Exp. Med.*, 1917, xxv, 557.

[2] Oliver, J., *J. Infect. Dis.*, 1922, xxx, 91.

[3] McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

[4] Wright, J. H., and Craighead, E. M., *J. Exp. Med.*, 1922, xxxvi, 135.

[5] Doerr, R., and Zdansky, E., *Schweiz. med. Woch.*, 1923, liii, 349.

[6] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Acad.*, 1923, clxxvii, 985.

[7] Cowdry, E. V., and Nicholson, F. M., *J. Am. Med. Assn.*, 1924, lxxxii, 545.

courtesy in allowing us to examine his preparations of rabbit brains; Dr. Brown, Dr. Pearce, and Dr. McCartney for allowing us to study, in cooperation with Dr. Olitsky, their extensive collection of rabbit material for parasites; and Dr. Laura Florence for her kindness in permitting us to study her sections of rabbit kidneys containing the parasites.

The Lesions.

The lesions (Figs. 1 to 6) were found in twenty-five out of a total of 132 mice examined histologically;[8] that is to say, in about 19 per cent. Eight of these twenty-five animals were to all appearances normal. The remainder had been used in the Institute for various experiments.[8] Focal infiltrations were most frequently met with and perivascular, meningeal, and subependymal infiltrations were seldom seen in their absence. It was, on the other hand, a common experience to find focal infiltrations when the other varieties were difficult to detect.

The infiltrations were observed in all parts of the brain from the tips of the olfactory lobes to the first cervical segment (and probably extend down the spinal cord), but they were encountered most frequently in the cerebral cortex and least frequently in the cerebellum. The perivascular infiltrations seemed particularly prone to occur in the hippocampus, while the subependymal proved to be more characteristic of the lateral ventricles than of the third or fourth ventricle.

[8] The mice were intentionally collected from a variety of sources in order to increase the likelihood of finding an infected strain. 78 of them were to all appearances normal and included specimens of different ages. The remaining 54 had been employed in the Institute in studies upon botulism, mouse typhoid, transplantable tumors, and for other experimental purposes. As a routine procedure we fixed the brains in Zenker's fluid (without acetic acid), and mounted as many serial sections on the slides as we could fit under a 24 by 60 mm. cover-glass. A few of the brains were cut into complete serial sections, while in the others serial sections were taken at the following levels: (1) olfactory bulbs, (2) optic chiasma, (3) corpora mammillaria, (4) corpus trapezoideum, and (5) decussation of the pyramids. The sections were usually colored with Giemsa's stain, but methyl violet and other special dyes were also used for the parasites, of which the fuchsin and methylene blue method advised by Wright and Craighead gave the most satisfactory results.

The use of serial sections and rough methods of reconstruction led to the observation that a definite topographic relationship exists between the meningeal, perivascular, and focal types. In many cases, both in the cerebral cortex as well as in the medulla oblongata, it was possible to trace complete continuity between a meningeal infiltration on the surface, its extension into the brain substance as a perivascular infiltration about a penetrating blood vessel, and its termination as a focal lesion either at the end of the vessel or to one side of it. For example, a meningeal infiltration of this kind penetrating along a blood vessel into the cortex is illustrated in Fig. 5. Serial sections showed that this perivascular lesion was directly continuous with a focal lesion situated more caudally. This gradation between perivascular and focal lesions is also indicated in Fig. 2. Subependymal lesions were likewise seen in connection with those of the perivascular variety. The perivascular infiltrations extended, in the majority of cases, along a considerable length of the vessels involved. Sometimes corresponding vessels on either side of the brain were found to be in sharp contrast, one being surrounded by a dense collar of cells, the other remaining entirely free from them.

The same kinds of cell were found to participate in the infiltrations quite irrespective of their location (meningeal, perivascular, focal, or subependymal). In all our specimens lymphocytes predominated, but a few plasma cells and macrophages were also noted. The latter were particularly numerous at the centers of the focal lesions in the presence of slight necrosis and in the absence of lymphocytes, which were clumped about the periphery (Fig. 8). In none of the lesions were polymorphonuclear leucocytes abundant. Very little cytoplasmic or nuclear débris was to be seen.

The youngest animal in which an infiltration was noted (Fig. 3) was only 2 days old, but we have some hesitancy in definitely including this lesion in our series as no parasites were found. Both lesions and parasites were abundant in another mouse of 3 weeks which was one from a litter of six. It was noted that none of the remaining five mice showed signs of infection, although they had lived from birth in the same jar with it under almost identical conditions. Similarly only one of a group of twenty mice, 1 month old, exhibited lesions in spite of the fact that the others were reared under

the same conditions and were presumably equally liable to infection. On the other hand, four mice which were kept for a short time in association (receiving doses of *botulinus* toxin) all showed the lesions and one of them parasites as well. But it was the exception rather than the rule for all mice held in close association under like conditions to exhibit these meningoencephalitic lesions. Both lesions and parasites were also observed in mature mice. We were unable to observe any definite clinical symptoms,—except in one case in which the lesions were localized in the cerebellum,—but it is possible that if the percentage involved had been a greater one, and if the mice had been held under observation for a longer period of time, some deviation from their normal behavior might have been detected.

The Parasites.

The parasites (Figs. 7 to 12) were detected in the brains of 20 per cent of the mice which exhibited the meningoencephalitic lesions and were never seen in brains in which the lesions could not be found. They were closely associated with all types of infiltration, except the meningeal, and it is not improbable that further search would have revealed them in the meninges also. Their much greater tendency to accumulate in focal areas rather than in prominent perivascular infiltrations,—where, however, they do occur (Fig. 11),—may be explained on the hypothesis that they escape more easily from the blood vessels of finer caliber. The presence of parasites is thus another point of correspondence between the different topographic varieties of infiltration. But many parasites were also found some distance away from the lesions and surrounded by fibers, nerve cells, neuroglia, and capillaries which showed no reaction at all to their presence. In these positions, however, it was observed that the parasites did not occur singly between the cells and fibers, or within the cytoplasm of macrophages, but were always clumped in fairly large masses walled off from the surrounding tissue by a membrane or pseudomembrane (Figs. 7, 9 to 12). These masses were also frequently noted about the periphery of areas of infiltration. In some of these "cysts," as they are called, in the rabbit, by Levaditi, Nicolau, and Schoen,[6] the parasites were closely wedged in together, while in others they were scattered apparently in suspension in a fluid which was evidently

under some pressure during life. As many as six cysts may occur in close apposition as represented in Fig. 7. One very characteristic element in this cyst formation was the presence at one side of the cyst of a large nucleus with a rim of cytoplasm which was greatly compressed and sometimes pycnotic. This is shown in Figs. 9, 10, and 12. From the size relations and the presence of large, solitary, and well formed nucleoli, it seemed clear that we were dealing with greatly distorted nerve cells. Unfortunately the evidence which we collected was conflicting as to whether, in these cases, the parasites were actually intracellular. We were not able to find what we could regard as early stages in the invasion of nerve cells, that is to say a variable number of parasites embedded in the cytoplasm in relation to the Nissl substance; but we did observe, on several occasions, a few scattered parasites close to the margins of the cells which raised the alternative hypothesis that the parasites might accumulate in the perineuronal spaces and, by distending them, bring about the pressure atrophy of the cells already alluded to. Against this view was the significant observation, derived through the study of serial sections, that there is no continuity between the contents of neighboring cysts, like those illustrated in Fig. 7, any more than there is between the contents of adjacent nerve cells; whereas the perineuronal spaces, unless greatly modified, would certainly exhibit some degree of continuity.

The shape of the parasites was found to be subject to some slight variation, but they were usually observed in the form of straight or slightly curved rods. Measured in stained preparations after Zenker fixation, these rods were, on an average, from 1.8 to 2 microns in length and from 0.5 to 0.8 micron in diameter. In other words, they were two or three times as long as they were broad. Both ends were bluntly and uniformly rounded. Very rarely were pear-shaped parasites seen, or forms with pointed ends. The parasites stained blue by Giemsa's method, pink with eosin, intensely red with fuchsin, and were somewhat resistant to decolorization by Gram's method.

They exhibited marked internal organization in the possession of a well defined mass of material which stained intensely by both acid and basic dyes. Usually this was of spherical shape and was located roughly at a point distant from one extremity by about one-third

TABLE I.

Comparison of Lesions and Parasites in Mice and Rabbits.

	Mice.	Rabbits.
Lesions.	Meningeal, perivascular, focal, and subependymal infiltrations of lymphocytes with a few plasma cells.	Similar meningeal, perivascular, and focal lesions noted by Oliver[2] and subependymal by McCartney.[5]
Distribution.	In all parts of the brain but particularly abundant in cerebral cortex and basal ganglia.	The same.
Incidence.	19 per cent in 132 (78 "normals" and 54 used for other experiments).	20 per cent of "normal" rabbits (Oliver). 55 per cent in 372 (52 "normals" and 320 used for other experiments) (McCartney).
Symptoms.	Usually absent.	None noticeable (Oliver). " " (McCartney). Motor paralysis (Wright and Craighead[4]).
Parasites.		
Size.	About 0.6 by 2 microns.	1.5 by 4 microns (Wright and Craighead). 1.5 by 3 microns (Doerr and Zdansky[5]). 1 to 2 microns (Levaditi, Nicolau, and Schoen[6]).
Shape.	Straight or slightly curved rods with both ends bluntly and uniformly rounded.	Sharply or smoothly outlined with rounded or conical ends (Wright and Craighead). Ovoid with one or both ends slightly swollen. Others sickle-shaped (Doerr and Zdansky). Oval, piriform, or seed-shaped (Levaditi, Nicolau, and Schoen).
Staining reactions.	Blue by Giemsa's method, pink with eosin, intensively red with fuchsin, and somewhat resistant to decolorization by Gram's method.	Color by Gram's method, with methylene blue, and are acid-fast to a certain extent (Wright and Craighead). Stain red by Ziehl-Neelsen fuchsin method (Doerr and Zdansky). Stain pale blue by hematin, strongly red by Mann's method, dark blue by polychrome methylene blue. Do not color by Gram or Ziehl (Levaditi, Nicolau, and Schoen).

TABLE I—*Concluded.*

	Mice.	Rabbits.
Internal organization.	A single nuclear-like mass at junction of middle and outer thirds, or two, one at each end.	One or two lightly staining areas (Wright and Craighead). Nuclear-like differentiations in middle or at either pole (Doerr and Zdansky). Protoplasmic body with grains of chromatin enclosed by a membrane (Levaditi, Nicolau, and Schoen).
Distribution.	Free and within macrophages in areas of infiltration as well as in cysts which, in addition to being associated with infiltrations, were found in other parts of the brain which exhibited no reaction to their presence. Cysts were often bounded to one side by single large flattened nuclei suggesting accumulation of the parasites within nerve cells.	Apparently exactly the same, even to the probable accumulation within nerve cells, as may be seen by comparing our illustrations with those of the above mentioned authors.[9]
	No lesions or parasites were seen in the kidneys although 216 mice were examined macroscopically for lesions and 20 microscopically for parasites.	Parasites noted in the kidneys by all of these three groups of investigators.
Incidence.	3.7 per cent in 132 mice. Never in animals without lesions.	2.6 per cent in 224 rabbits, never in animals without lesions (Doerr Zdansky[10]). 1.5 per cent in 200 rabbits examined by us, in cooperation with Dr. Olitsky, but never in animals without lesions.[11]

[9] Oliver expresses himself as unable to decide the exact nature of these 'cysts' although they appeared in his preparations "to be simply cavities formed by the destruction of the nerve tissue and surrounded by the proliferation of cells" (Oliver, J., *Arch. Neurol. and Psychiat.*, 1924, xi, 321).

[10] Doerr, R., and Zdansky, E., *Z. Hyg. u. Infectionskrankh.*, 1923, ci, 239.

[11] Further data regarding the disease in rabbits are given by Goodpasture, E. W., *J. Infect. Dis.*, 1924, xxxiv, 428.

of the total length of the parasite, as is clearly represented in a parasite near the left-hand margin of Fig. 7 and in another slightly to the right of the center of Fig. 8. Occasionally this nuclear-like mass was divided into two parts, one located at either end of the parasite. Sometimes approximately one-half of the parasite stained strongly, the other half very lightly. In all cases a fairly definite membrane limited its protoplasmic contents. This membrane was most readily visible after staining by Giemsa's method.

Comparison of Lesions and Parasites with Those Reported in the Rabbit.—The parasites called to mind so forcibly certain photographs published by Wright and Craighead of protozoan-like organisms in young rabbits, which we have already referred to, that we sent some of our specimens to Dr. Wright who expressed himself as convinced that the parasites which we have found in mice are the same as those which he and Craighead discovered in rabbits.[12] We have set forth in Table I a comparison of both lesions and parasites in the two animals.

DISCUSSION.

Evidently the association between the presence of protozoan-like parasites and of infiltrative lesions which has recently been discovered in the rabbit brain, in the absence of any distinctive clinical symptoms, also obtains in mice and perhaps in other laboratory animals.[13]

Levaditi, Nicolau, and Schoen[6] are of the opinion that, in rabbits, the parasites cause the lesions and propose the term "*Encephalitozoon*

[12] Levaditi, Nicolau, and Schoen have recently (Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Soc. biol.*, 1924, xc, 662) confirmed our observation[7], that the parasites, first found in rabbits, occasionally occur also in mice.

[13] Reference is here intended to the lesions reported in monkeys by Lucke, B., *Arch. Neurol. and Psychiat.*, 1923, x, 212.

Since this paper was submitted for publication, the brains from a series of 105 guinea pigs and 114 white rats have been surveyed systematically. Selection of animals and fixation, staining, serial sectioning, and examination of tissues for lesions and parasites were similar to those described for the study of the rabbits and mice herein given. Of the 105 guinea pigs, four, or 0.038 per cent, were found to have lesions. Three of them showed focal and one meningeal infiltrations. Focal infiltration was observed in but one, 0.0087 per cent, of the 114 rats. In none of them were there found parasites like those associated with lesions in the other animals.

cuniculi," to indicate this hypothesis. As evidence, they present the observation that the lesions and parasites are to be found in the brains of rabbits which have received inoculations with the viruses prepared by Kling, by Thalhimer, and by other investigators. Unfortunately their experiments are not published in detail but we note that they allowed[14,15] a period of from 100 to 150 days to elapse between the time of inoculation and the date when the animals were finally sacrificed and examined microscopically. Since we know, from the observations of Wright and Craighead, that the parasites escape with the urine, and are presumably taken in with the food, there is the possibility that infection took place independently of the inoculations, unless Levaditi, Nicolau, and Schoen were careful to select the rabbits from a stock proved to be free from lesions and parasites, and unless they maintained a strict quarantine during the experiments.

From the fact that laboratory mice are infested by the same parasites—or by others belonging to a nearly related species, because slight differences may be brought to light when the life cycles become known—it follows that similar difficulties will be encountered in ascertaining the precise relationship between the parasites and the lesions. In both animals the parasites are less frequently met with than the lesions, which calls to mind the observation by Smith and Johnson[16] of somewhat similar infiltrations in the kidneys of mice which they considered to be old foci of invasion by *Klossiella muris* and later abandoned by them.[17] Since the parasites were originally discovered by Wright and Craighead in young rabbits and we have found them in mice only 3 weeks old, it is possible that, like *Klossiella muris*, they may excite lesions which persist after their own disappearance. This would explain the large percentage of adults showing lesions as contrasted with the small percentage exhibiting parasites.

[14] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Soc. biol.*, 1923, lxxxix, 984.

[15] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Soc. biol.*, 1923, lxxxix, 1157.

[16] Smith, T., and Johnson, H. P., *J. Exp. Med.*, 1901–05, vi, 303.

[17] We are indebted to Dr. Smith for the information that the parasites with which we are concerned are not *Klossiella muris*.

But from the standpoint of histopathology the lesions are not very distinctive. They have the appearance of being chronic and resemble in some respects the infiltrative lesions of syphilis and of lethargic encephalitis as well as others recently reported by Paulus,[18] by Semerak,[19] and by Warthin,[20] under the heading of botulism. Though the parasites may be capable of producing them, and probably actually do so in many cases, it must be admitted that the evidence at hand does not preclude the possibility of the operation of other factors; for it is quite conceivable that infiltrative lesions of this general category, in which lymphocytes predominate, may arise in more than one way. The laboratory animals concerned are subject to several diseases which have not as yet been definitely excluded as causal factors. McCartney's observation that the lesions are present in 76 per cent of rabbits ill with snuffles as compared with an incidence of 55 per cent in apparently healthy animals indicates that we may have several influences at work simultaneously—in other words that the lesions may be more likely to appear in animals whose vitality is temporarily reduced. McCartney has further noted that the lesions are more numerous in rabbits "suffering from miscellaneous diseases such as pneumonia, septicemia, and subcutaneous abscesses."

Our experience has been the same with mice. We found lesions in 10 per cent of "normal" animals and in 31 per cent of animals employed in the laboratory for different experimental purposes.[8] In connection with this discussion of etiology mention may also be made of the suggestion that the lesions may be caused by the action of a filter-passing virus.[21,22]

A peculiar fact, noted by Oliver, is that the disease in rabbits has apparently not been of constant duration. He found, for example, lesions in 20 per cent of the rabbits examined in 1921, although Dr. E. C. Dickson did not meet with them in 60 rabbits from the same stock room, examined in 1917. It is interesting also that Kling,

[19] Paulus, E. A., *J. Psychol. u. Neurol.*, 1915, xxi, 201.
[20] Semerak, C. B., *J. Infect. Dis.*, 1921, xxix, 190.
[21] Warthin, A. S., *Tr. Assn. Am. Phys.*, 1922, xxxvii, 357.
[22] Twort, C. C., *Vet. J.*, 1922, lxxviii, 194.
[23] Twort, C. C., and Archer, H. E., *Lancet*, 1923, i, 1102.

Davide, and Liljenquist[23] examined 196 rabbits in Stockholm without finding any trace of encephalitis. This lends color to the theory that the disease in mice, as well as in rabbits, living under laboratory conditions, may be of relatively recent origin and in turn offers a plausible explanation of the failure of earlier workers to observe lesions which are so noticeable and parasites which are so large and easily stained. The more is this likely since these two animals have been subjected to the close and constant scrutiny of neurologists working upon brain architecture in all parts of the world for nearly a century. A natural line of inquiry is to be found in the study of wild rabbits and mice but no progress has been reported in this direction.

CONCLUSIONS.

A small proportion of laboratory mice, which appear to be normal, are in reality suffering from an obscure disease characterized by the presence of marked meningoencephalitic lesions which are often accompanied by protozoan-like parasites.

Both the lesions and the parasites closely resemble others previously reported in rabbits, which likewise fail to reveal themselves by pronounced clinical symptoms.

In the absence of direct experimental evidence it is suggested either that one species of parasite is capable of producing the lesions in both animals, or else that we have to do with two kinds of parasites which are closely related and, in the stages thus far observed, are indistinguishable one from the other.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Small focal infiltration in the thalamus of an adult white mouse (No. 19) stained 53 days previously with trypan blue *intra vitam*. Zenker, Giemsa. × 220.

FIG. 2. Focal infiltration in continuity with adjacent perivascular infiltration. Same mouse and technique. × 122.

FIG. 3. Small area of infiltration into peripheral layer of brain substance in a mouse 2 days old (No. 2). Zenker, Giemsa. × 220.

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[23] Kling, C., Davide, H., and Liljenquist, F., *Compt. rend. Soc. biol.*, 1924, xc, 507.

FIG. 4. Perivascular infiltration in the temporal cortex of an adult mouse (No. 114). Zenker, Giemsa. × 450.

FIG. 5. Meningeal infiltration continuous with a penetrating perivascular infiltration which in turn passes into an area of focal infiltration, only a trace of which may be seen in this photograph, although it is well marked in the next section. Adult white mouse (No. 81). Zenker, Giemsa. × 122.

FIG. 6. Small area of focal infiltration in the molecular layer of the cerebellum of a white mouse 3 weeks old (No. 20). Zenker, Giemsa. × 450.

PLATE 4.

FIG. 7. A collection of six cysts packed with parasites in the cerebral cortex of a mouse 3 weeks old (No. 20). Along the left-hand margin of the photograph something may be distinguished of the internal structural organization of the parasites. Zenker, fuchsin, and methylene blue. × 1,500.

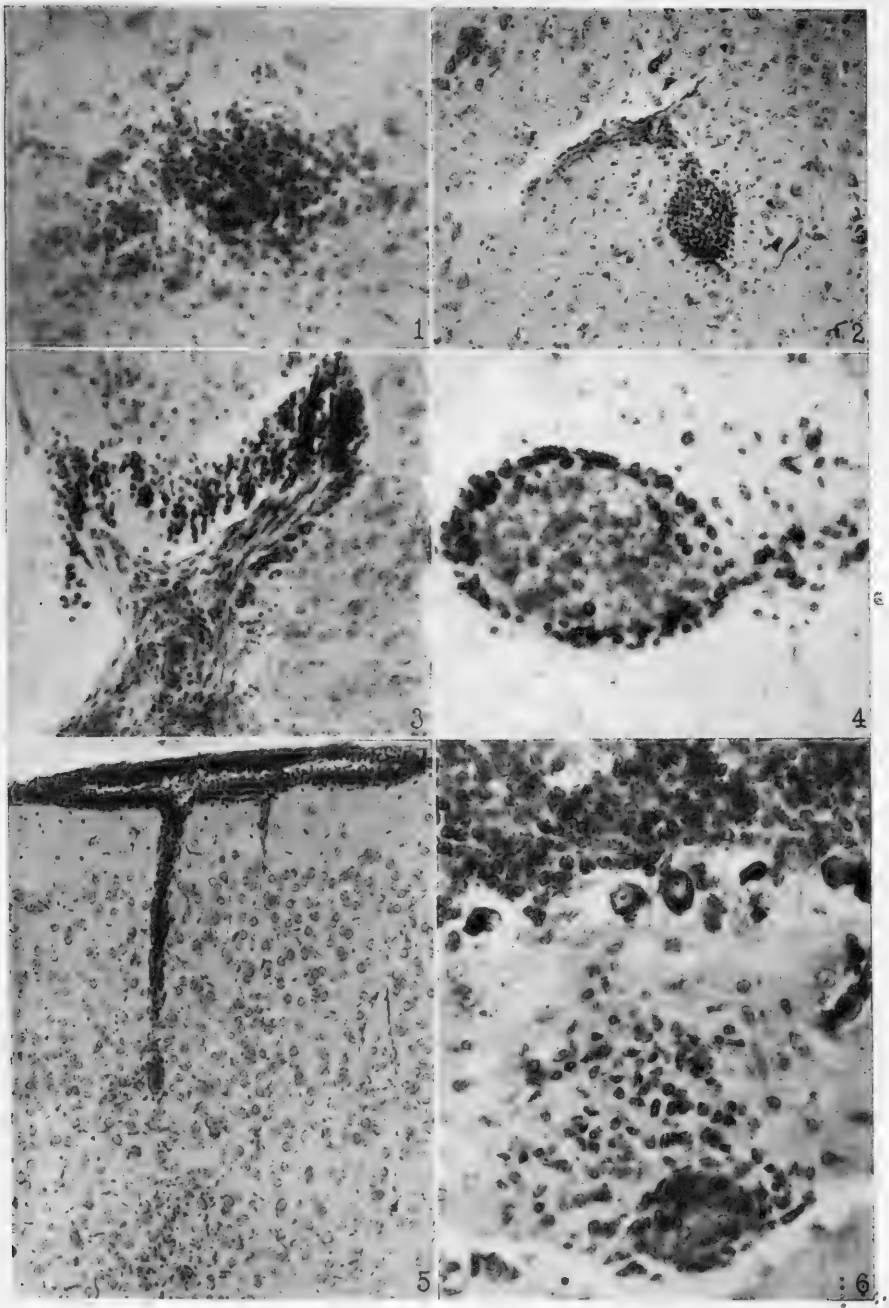
FIG. 8. Scattered parasites within the cytoplasm of macrophages in a focal area of infiltration in the cortex of a young adult mouse (No. 114). Zenker, fuchsin, and methylene blue. × 1,500.

FIG. 9. Mass of parasites with adjacent compressed cell, rather pycnotic, with a distinct nucleus and nucleolus in the medulla at the level of the nucleus of the seventh nerve of a mouse 3 weeks old (No. 20). Zenker, Giemsa. × 1,000.

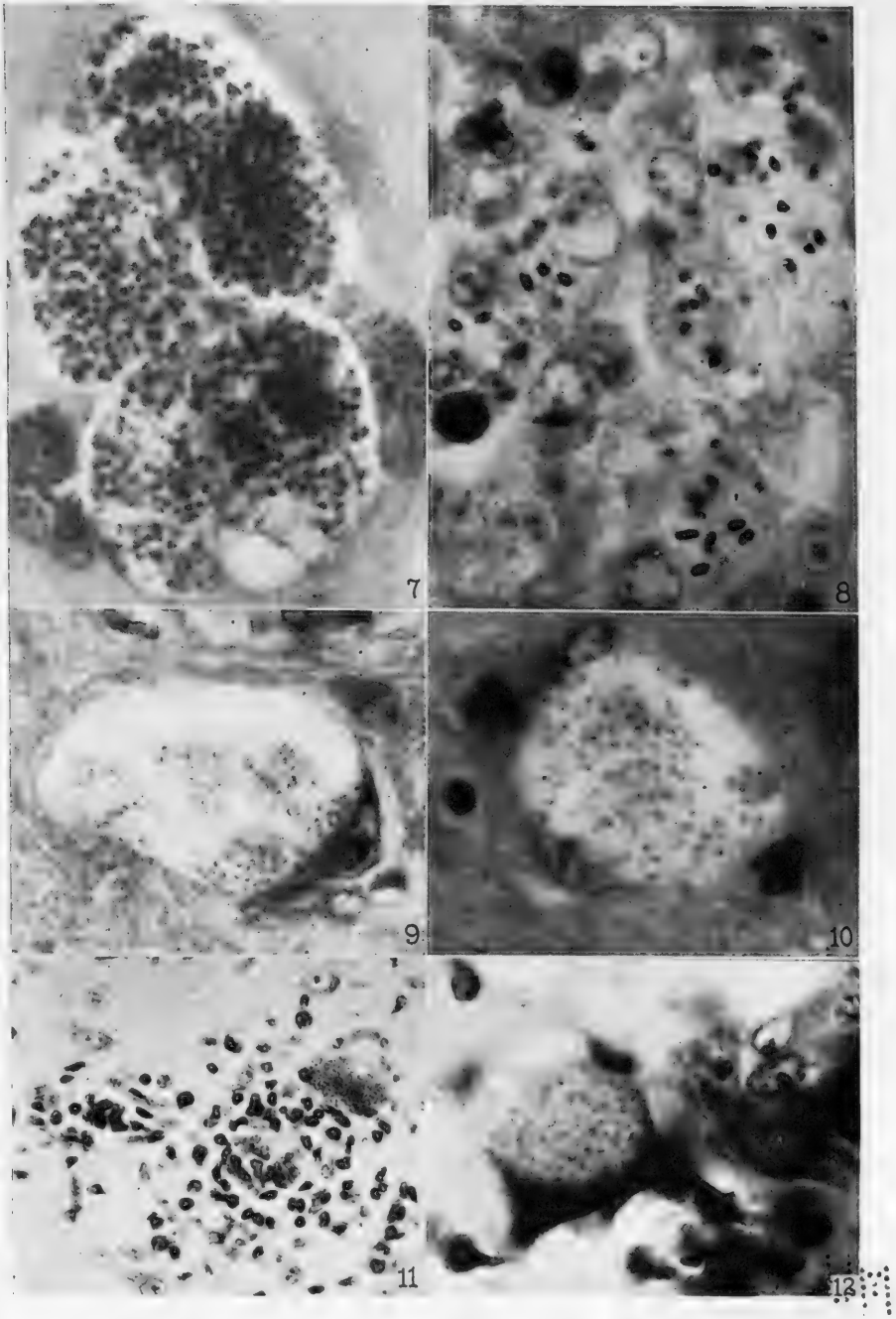
FIG. 10. A similar cyst with compressed cell in the brain stem at the level of the inferior colliculus of a mouse 3 weeks old (No. 20). Zenker, Giemsa. × 1,500.

FIG. 11. A cyst packed with parasites in association with a perivascular lesion in the brain stem at the level of the inferior colliculus in a mouse 3 weeks old (No. 20). Zenker, Giemsa. × 450.

FIG. 12. A cyst containing many parasites in close apposition to a focal area of infiltration in the medulla of an adult mouse (No. 81). Zenker, Giemsa. × 1,500.



(Cowdry and Nicholson: Meningoencephalitis.)



(Cowdry and Nicholson: Meningoencephalitis)

THE EFFECTS OF THE INTRATRACHEAL ADMINISTRATION OF FOREIGN SERUM.

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In the course of certain investigations rabbits repeatedly injected with bovine serum by the intraperitoneal and intratracheal routes were tested for sensitiveness. It was then found that an animal sensitized by intraperitoneal injections failed to react on subsequent intratracheal injection. Another sensitized by intratracheal injections promptly reacted to a small amount administered intravenously. It had been established by the experiments of Pfenninger,[1] D'Aunoy,[2] and Jones[3] that antibodies are readily formed in rabbits after intratracheal injections of suitable antigens. Precipitin production is particularly marked in animals treated by intratracheal injections of foreign serum. Besredka[4] had considered the question of sensitization through the lower respiratory route. He noted that guinea pigs sensitized with small doses of horse serum or egg white when reinjected into the trachea with small quantities of the antigen developed anaphylactic shock. It was stated that the trachea easily absorbs large quantities of serum. He further concluded that the rapidity of absorption, the absence of danger of anaphylactic shock, and the simplicity of the technique made the laryngotracheal method suitable for serum therapy in the case of man.

The inability of the writer to produce shock on intratracheal injection in the case of the rabbit was of considerable interest. It seemed possible to throw some light on the absorption of protein through the lower respiratory tract by continuing the observations along similar

[1] Pfenninger, W., *Ann. Inst. Pasteur*, 1921, xxxv, 237.
[2] D'Aunoy, R., *J. Infect. Dis.*, 1922, xxx, 347.
[3] Jones, F. S., *J. Exp. Med.*, 1923, xxxvii, 789.
[4] Besredka, A., *Ann. Inst. Pasteur*, 1920, xxxiv, 51.

lines. Inasmuch as the guinea pig is extremely sensitive to injections of protein it was decided to use this species. With a little practice doses as high as 2 cc. may be introduced by means of a curved metal tube passed through the larynx by way of the mouth into the trachea.

EXPERIMENTAL.

It was desirable to determine whether guinea pigs could be sensitized by the administration into the trachea of small doses of serum. The method of making intratracheal injections employed throughout the experiments is essentially the same as that previously described.[3] All animals were given sufficient ether to cause complete relaxation and as little pressure as possible was employed in making the injections.

In the first experiments one-half of the animals received intratracheal injections of horse or cow serum and the remainder were sensitized intraperitoneally. After sufficient time had elapsed, one-half of the animals of each series were then injected intravenously with 0.5 cc. of a 40 per cent solution of serum. They promptly succumbed to fatal anaphylactic shock. The remainder were treated with similar doses intratracheally. All remained well. The results were so strikingly different from those recorded by Besredka that further experiments were undertaken.

Experiment 1.—Six guinea pigs averaging 500 gm. in weight were divided into two groups. Three were sensitized by the intratracheal route and the remainder by the intraperitoneal route. Two injections of 0.5 cc. of 40 per cent horse serum were administered on Jan. 4 and Jan. 6. On Jan. 20, all were injected with 1 cc. of horse serum. The results are given in Table I.

This experiment was repeated a number of times. In two series bovine serum was employed. The results were essentially the same. It was possible to sensitize by the intratracheal injection of small quantities of foreign serum. Evidently sufficient protein is absorbed through the alveoli and air passages to serve for the purpose of sensitization. Yet serum in the indicated doses when introduced into the trachea of sensitized animals is evidently incapable of giving rise to marked anaphylactic phenomena, whereas similar doses injected into the peritoneal cavity led to well marked reactions.

It might be argued that the serum introduced into the lower respiratory tract was forcibly expelled before there was time for its absorption, although animals properly anesthetized rarely cough after injection. In answer to such objection and in order to ascertain whether by a

TABLE I.

No. of guinea pig.	Method of sensitization.	Tested by injection of 1 cc. of serum.	Result.	Remarks.
1	Intratracheal route.	Intratracheally.	No reaction.	
2	" "	Intraperitoneally.	Died in 30 min.	Anaphylaxis.
3	" "	Intratracheally.	No reaction.	
4	Intraperitoneal "	"	" "	
5	" "	Intraperitoneally.	Severe reaction in ½ hr. Recovered.	Temperature fell to 32.-7°C.
6	" "	Intratracheally.	No reaction.	

TABLE II.

No. of guinea pig.	Method of sensitization.	Tested by injection of serum.		Result.	Remarks.
		Amount.	Route.		
		cc.			
7	Intratracheal route.	2	Intratracheal.	No reaction.	
8	" "	1	Intraperitoneal.	Severe shock in 15 min.	Temperature fell to 35°C. Recovered.
9	Intraperitoneal "	2	Intratracheal.	Moderate shock.	Temperature 36.-4°C. Recovered.
10	" "	1	Intraperitoneal.	" "	Temperature 35.-9°C. Recovered.
11	" "	2	Intratracheal.	No reaction.	

reasonable dose into the trachea shock could be brought on, animals were first treated as in Experiment 1. 14 days after the second sensitizing injection the tests were made. The details are given in Table II.

It will be observed that the results were in the main similar to those recorded in Table I. The introduction of 2 cc. of serum into the trachea of sensitive guinea pigs resulted in a mild shock in one instance. The other two animals were unaffected. 1 cc., half the dose given intratracheally, when injected into the peritoneal cavity was sufficient to produce characteristic disturbances. 2 cc. is a relatively large amount to inject into the trachea. While the animal can tolerate this amount of liquid without difficulty, nevertheless the operator is hurried and must inject with more force than is usually employed. That certain portions of the lung may have been injured during the injection is possible, and such injury would probably facilitate a rapid absorption of the serum.

A similar experiment in which three guinea pigs were sensitized by the intraperitoneal injection of bovine serum and tested by the intratracheal route gave substantially the same results. One of the guinea pigs was given 1 cc. of serum into the trachea. It remained well. The second received 1.5 cc. It also failed to react. The third treated with 2 cc. of serum intratracheally developed a characteristic shock and died within 45 minutes.

There seemed to be three possible explanations for the failure of moderate doses of serum to induce shock in the sensitive animal; *viz.*: (1) The intact lining membranes of the alveoli and air passages may be relatively impermeable to colloidal substances. (2) Absorption may go on so slowly that insufficient serum gains access to the blood at one time to produce shock. (3) The substances injected may not remain in contact with the lining membranes long enough to be absorbed.

The lining of the air spaces is an exceedingly thin layer of epithelial cells, supported by a delicate membrane and should afford one of the most permeable mucous membranes of the body. It seemed possible to judge the rapidity of absorption of foreign protein by the following experiment.

Experiment 3.—Four rabbits were injected with bovine serum. In Rabbits 1 and 2 the serum was administered by way of the trachea. It was injected into the peritoneal cavities of Rabbits 3 and 4. Each rabbit was bled 3 cc. from the ear vein just before injection and at hourly intervals subsequently. Precautions were taken to avoid hemolysis. The rabbit serum after it had exuded from the clot

was centrifuged so that clear, straw-colored samples were obtained. The various samples of serum were tested with the serum of a rabbit that had been immunized with bovine serum. It had been ascertained that 0.1 cc. of this precipitin would detect 1/10,000 cc. of bovine serum. The results are given in Table III.

TABLE III.

*The Rapidity of Absorption of Foreign Serum into the Blood Stream after Intratracheal and Intraperitoneal Injection.**

Time serum was obtained.	Rabbit 1. Weight 2,618 gm. 6 cc. of serum injected intratracheally.				Rabbit 2. Weight 2,752 gm. 8 cc. of serum injected intratracheally.				Rabbit 3. Weight 2,724 gm. 6½ cc. of serum injected intraperitoneally.				Rabbit 4. Weight 2,530 gm. 6 cc. of serum injected intraperitoneally.			
	Amount of serum tested.				Amount of serum tested.				Amount of serum tested.				Amount of serum tested.			
	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.
Before injection.	−			−		−	−	−	−	−		−		−		
After " †																
hrs.																
1	−			−		−	−	−		+	+	+		+		
2	−	−				−	−	−		+	+	+		+		
3	−	−		−		?	−	−		+	+	+		+		
4	−	−		−		+	−	−		+	+	+		+		
5	±	±		−		+	±	−		+	+	+		+		
6	+		+	−		+	+	−		+	+	+		+		
7	+	+		−												
8	+	+	−	−		+	+	+								
24	+	+		±												

* Definite precipitation has been recorded as +; ± indicates a slight turbidity without recognizable precipitate.

† Strong reactions were obtained in all three tubes of serum obtained ½ hour after injection in Rabbits 3 and 4.

The rapidity of the absorption into the blood stream of the serum from the peritoneal cavity is noteworthy in the rabbits (Nos. 3 and 4) injected intraperitoneally. Within ½ hour strong precipitations were obtained in the sera from the peripheral blood. Such was not the case when comparable doses were injected into the trachea, for the foreign serum could not be definitely detected in the blood until 4 or 5 hours after the injection. It was possible to judge the amount of absorption by the intensity of the reactions in the test-tubes. The

reactions in the instances of Rabbits 3 and 4 were uniformly much more marked throughout the series. Evidently absorption from the peritoneal cavity is more considerable as well as more rapid than that from the lower respiratory tract.

It has thus been shown both by the anaphylactic reactions and the precipitin tests that foreign proteins are absorbed slowly and in small quantities through the lower respiratory tract. That the quantities absorbed are on the whole insufficient to produce anaphylactic phenomena is equally clear. The question whether the serum is maintained in contact with the membranes for a sufficient period to be absorbed must be considered.

TABLE IV.

No. of guinea pig.	Interval after intratracheal injection with bovine serum animals were killed.	Results of tests with precipitin specific for bovine serum.					
		Tracheal and bronchial washings.			Lung extract.		
		Amount tested.			Amount tested.		
		1.0 cc.	0.1 cc.	0.01 cc.	1.0 cc.	0.1 cc.	0.01 cc.
	hrs.						
12	1	+	+	−	+	−	−
13	3	+	+	−	+	+	−
14	6	+	+	+	+	+	−

To throw some light on this point, three guinea pigs were each injected intratracheally with 1 cc. of cow serum. 1, 3, and 6 hours after injection they were anesthetized with ether and the vessels of the neck severed. The lungs were removed as quickly as possible and 5 cc. of salt solution injected into the trachea. After a short interval the base of the lungs was elevated and as much of the salt solution collected as possible. In addition small pieces of the more dependent portions of all lobes were ground in a mortar with sand and triturated with 5 cc. of salt solution. Both the tracheal washings and the ground suspensions were filtered through filter paper. After refrigeration overnight, all fluids were centrifuged rapidly and the supernatant liquid tested for cow serum with the same precipitin used in the previous experiment. The results are given in Table IV.

The table indicates that at least a portion of serum remains in the air passages for a considerable period since the tracheal washings and lung extracts gave the characteristic precipitation even as long as 6 hours after the injection. It is obvious then that the injected material is not promptly eliminated from the lower respiratory tract. Evidently it is in contact with the membrane for a sufficient period to permit absorption. It becomes apparent then that physical characters of the membranes limit the rate and the amount of absorption.

Many of the guinea pigs used in Experiments 1 and 2 survived. A good opportunity was afforded to ascertain to what degree the intratracheal injection had influenced the degree of sensitiveness. The

TABLE V.

No. of guinea pig.	First test injection.		Second test injection, 1 cc. intraperitoneally.	
	Route.	Reaction.	Reaction.	Minimum temperature.
1	Intratracheal.	None.	Shock.	36.2°C.
3	"	"	"	36.3 "
4	"	"	"	35.9 "
5	Intraperitoneal.	Severe shock.	None.	
6	Intratracheal.	None.	?	37.0 " a fall of 1.8°C.
7	"	"	None.	
8	Intraperitoneal.	Shock.	"	
9	Intratracheal.	"	"	
10	Intraperitoneal.	"	"	
11	Intratracheal.	None.	Fatal shock.	

survivors of Lot 1 were retested 2 days after the first test; those of Lot 2 after 4 days. All animals of both series were injected intraperitoneally with 1 cc. of horse serum. The results are recorded in Table V.

The results appear definite. Guinea Pigs 1, 3, 4, 6, and 11 were first tested by intratracheal injection. None reacted. The second test, made by injecting 1 cc. of serum into the peritoneal cavity shortly after the first test, resulted in characteristic reactions in four animals. The reaction in the other (No. 6) was indefinite. It presented no special symptoms other than a rapid fall in temperature within an hour following the injection. This reaction continued for $3\frac{1}{2}$ hours.

The temperature became normal 3 hours later. Of the four (Nos. 5, 8, 9, and 10) which showed symptoms of anaphylaxis following the first injection, all were refractory to the second dose. No. 7, which failed to react after the administration of 2 cc. of serum intratracheally, was able to withstand the second dose without manifesting symptoms. In this instance it is assumed that sufficient protein was absorbed as the result of the first injection to render the animal insensitive to the second dose.

DISCUSSION.

The experimental evidence indicates that animals become sensitive to proteins administered by way of the trachea, since the animals injected by that route and later treated with serum developed shock. The results of certain experiments indicate that the rate of absorption of serum after intratracheal injection is relatively slow. There are further indications that the amount of absorption is limited. The experiment in which a large quantity of serum was injected into the peritoneal cavities of two rabbits and into the tracheas of two others and the blood subsequently tested for the foreign serum at regular intervals seems clearly to demonstrate these points. The blood of the rabbits which received the serum into the peritoneal cavity contained the foreign serum in recognizable quantity within a half hour. It took considerably longer ($3\frac{1}{2}$ to $4\frac{1}{2}$ hours) for the foreign serum to reach the blood in the instances of those injected intratracheally. The intensity of the reactions in the test-tubes suggests that little of the injected protein reaches the blood in the case of the animals which received an intratracheal injection. This is further borne out by the protocols given in Table V, since four of the five animals which failed to react when serum was introduced into the trachea developed characteristic symptoms of anaphylaxis after subsequent intraperitoneal treatment with small doses. Evidently the absorption was so limited after intratracheal injection that the guinea pigs still remained sensitive to the relatively small test doses.

These facts indicate that the lining membranes of the alveoli and air passages permit a slight absorption of the foreign protein. Wells and Osborne[5] have shown that exceedingly minute amounts of protein will induce susceptibility.

[5] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

The hypothesis that the intact epithelium in itself affords a relatively impermeable barrier to colloids is further strengthened by the inability to produce shock by the intratracheal route in sensitive individuals with doses sufficient to bring on a characteristic reaction when introduced into the peritoneal cavity or into the circulation. It is true when the dose was doubled sufficient serum was absorbed to bring on characteristic symptoms in a limited number of animals. It was, however, pointed out that in such injections the operator is hurried and employs more force in making the injection so that injury leading to rapid absorption cannot be excluded as a complication. In addition the results of a considerable number of other experiments[6] dealing with the passage of antibodies from the lower respiratory tract into the circulation indicate that the lining membranes do not permit the passage of agglutinins and hemolysins in measurable quantities.

SUMMARY.

It has been possible to sensitize guinea pigs by the intratracheal administration of small amounts of horse or cow serum. The degree of sensitiveness is comparable to that obtained when the same doses of serum are injected into the peritoneal cavity. Nevertheless, relatively small amounts of foreign protein are slowly absorbed through the lining membranes of the lower respiratory tract. In sensitive animals it has not been possible to produce shock by intratracheal injection of amounts of serum which injected into the peritoneal cavity of other animals of the same series sensitized in the same manner always produced shock. Considerably larger doses administered intratracheally may give rise to anaphylactic symptoms. It is possible that the force necessary to inject the larger amounts of serum may have led to injury and more rapid absorption.

[6] Jones, F. S., *J. Exp. Med.*, 1924, xl, 73.

THE PERMEABILITY OF THE LINING OF THE LOWER RESPIRATORY TRACT FOR ANTIBODIES.

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In a previous communication[1] it has been shown that the membranes of the lower respiratory tract permit the absorption of proteins to a limited degree. In the course of experiments to ascertain to what extent the mucous membrane of the trachea, air passages, and alveoli would permit the passage of serum, foreign sera rich in antibodies were introduced into the trachea. Since the laryngotracheal administration of antisera as a practical procedure has been suggested by Besredka,[2] it seemed desirable to record the results of some observations by the writer. Besredka found that rabbits and guinea pigs can be protected against tetanus and diphtheria toxin by first administering concentrated antitoxin intratracheally. Levaditi[3] points out that an intratracheal injection of serum from a convalescent may stop short an attack of poliomyelitis.

Inasmuch as the writer's experience has been that foreign serum is absorbed slowly and in relatively small quantities, it seemed reasonable to infer that the antibodies contained in the serum would not penetrate the membranes to a greater degree. That this is the case is shown in the following series of experiments.

EXPERIMENTAL.

Inasmuch as the absorption of agglutinin is easily measured, in most of the experiments serum rich in agglutinin was introduced into the trachea by the method previously described.[4] The control ani-

[1] Jones, F. S., *J. Exp. Med.*, 1924, xl, 63.
[2] Besredka, A., *Ann. Inst. Pasteur*, 1920, xxxiv, 51.
[3] Levaditi, C., Ectodermoses neurotropes, Paris, 1922.
[4] Jones, F. S., *J. Exp. Med.*, 1923, xxxvii, 789.

mals received a comparable amount of the same serum intraperitoneally. In the observations dealing with the passage of agglutinin, the animals were bled at regular intervals and the collected serum tested with the original antigen.

In the first series of experiments cow serum containing agglutinins for the non-hemolytic mastitis streptococcus was employed. It agglutinated suspensions of the streptococcus at a dilution of 1:10,000.

TABLE I.

The Absorption of Agglutinin in Cow Serum Administered into the Trachea and Peritoneal Cavity.

Time serum was obtained.	Rabbit 1. Weight 2,618 gm. 6 cc. of bovine streptococcus serum injected intratracheally.				Rabbit 2. Weight 1,960 gm. 5 cc. of bovine streptococcus serum injected intratracheally.				Rabbit 3. Weight 2,530 gm. 5.8 cc. of bovine streptococcus serum injected intraperitoneally.					
	Results of tests of rabbit sera for the presence of agglutinins specific for a bovine streptococcus.													
	Serum dilutions.				Serum dilutions.				Serum dilutions.					
	1:20	1:50	1:100	1:200	1:20	1:50	1:100	1:200	1:20	1:50	1:100	1:200	1:500	1:1,000
Before injection.	−	−	−	−	++	−	−		++	+	−	−		
After injection. *hrs.*														
1	++	+	−	−	C.	C.	±	−		C.	++++	+++	++	+
2	++++	±	−	−	+++	−	−	−		"	C.	++++	+	−
3	C.	−	−	−	+	−	−	−		"	++++	+++	+	+
4	++	−	−	−	+++	−	−	−		"	C.	+++	+	+
5	+	−	−	−	+++	+	−	−		"	++++	+++	+	+
6	+	−	−	−	+++	+	−	−		"	++++	+++	+	+

The results following injections of this serum into the trachea and peritoneal cavity are given in Table I.

The results of the various tests indicate that the antibody injected into the trachea is absorbed into the circulation in relatively small amounts. Assuming that all the serum injected into the rabbit is absorbed during the course of the experiment, a strong agglutination should result when the serum of the animal is diluted 200 times. Such is not the case in the rabbits (Nos. 1 and 2) treated intratracheally.

The titer of the serum of the control rabbit (No. 3) injected intraperitoneally approaches the theoretical figure and indicates that the maximum absorption has taken place within 2 hours following the injection. In a similar experiment in which bovine serum rich in agglutinins for *Bacillus abortus* was injected intratracheally, the agglutinins for *Bacillus abortus* were not appreciably increased in the blood stream.

Confirmatory results were obtained when serum hemolytic for rabbit red cells was introduced into the trachea and peritoneal cavity. The hemolytic serum was obtained by injecting suspensions of washed red blood cells of the rabbit into the peritoneal cavities of guinea pigs. After sera sufficiently high in hemolysin had been obtained the guinea pigs were bled. The sera of all the guinea pigs were mixed and injected into the trachea or the peritoneal cavity of rabbits. The effect of the serum was measured by hemoglobin estimations and red cell counts at intervals following the injections. The details of the methods of administration and the effects upon the red cells *in vivo* are given in Table II.

The rabbits injected intratracheally with the hemolytic serum failed to react to a measurable degree, for one can scarcely admit as evidence the transient diminution in the number of red cells of Rabbit 5. Both rabbits survived and suffered no appreciable ill effects. In sharp contrast to these results are the marked effects following intraperitoneal injection. The red cells of Rabbit 4, which was injected intraperitoneally with 6.5 cc. of serum, began to decrease $3\frac{1}{2}$ hours after injection. The hemoglobin remained stationary during the first $6\frac{1}{2}$ hours. By the 23rd hour the anemia was severe. Rabbit 7, which received 3.5 cc. of serum intraperitoneally, ultimately reacted in a similar manner. It is of interest to note that in this instance there was an apparent increase in the red cells and hemoglobin during the first 6 hours following injection. At this time the capillaries of the ears were dilated and the blood was almost black in color.

It becomes evident that antibodies contained in a heterologous serum are not appreciably absorbed through the walls of the lower respiratory tract. The possibility suggested itself that antibodies contained in a homologous serum might be more readily absorbed.

TABLE II.

No. of rabbit.	Time blood was examined.	Hemoglobin.	Red cells.	Remarks.
	Before injection.	90	6,000,000	6.5 cc. of hemolytic serum injected intraperitoneally.
	After "			
	hrs.			
	½	92		
4	1	90		
	1½	89	6,048,000	
(Weight	2½	87		
2,400 gm.)	3½	90	5,240,000	
	5½		4,600,000	
	6½	90	4,528,000	
	23	35	1,304,000	
	31	25	1,248,000	
	48	25	752,000	Died 56 hrs. after injection.
	Before injection.	68	4,400,000	6.5 cc. of hemolytic serum injected intratracheally.
	After "			
	hrs.			
	½	67		
	1	68		
5	1½	68		
	2½	68		
(Weight	4		3,928,000	
2,400 gm.)	5	67	4,330,000	
	6	67	4,172,000	
	23½	68	4,172,000	
	31	68	3,976,000	
	48	68	4,000,000	
	72	67	4,200,000	Rabbit failed to show symptoms.
	Before injection.	80	4,172,000	7.0 cc. of hemolytic serum injected intratracheally.
	After "			
6	*hrs.*			
	1½	80	4,528,000	
(Weight	3	90	5,132,000	
2,195 gm.)	4½	85	5,128,000	
	6	83	5,088,000	
	24	80	4,784,000	
	31	90	5,004,000	Rabbit failed to show symptoms.

TABLE II—*Concluded.*

No of rabbit.	Time blood was examined.	Hemoglobin.	Red cells.	Remarks.
	Before injection.	80	4,880,000	3.5 cc. of hemolytic serum injected intraperitoneally.
	After "			
7	*hrs.*			
	1½	90	5,432,000	
(Weight	3	90	5,632,000	
2,210 gm.)	4½	107	6,304,000	
	6	108	6,208,000	
	24	52	2,768,000	
	31	46	2,008,000	Died 60 hrs. after injection.

TABLE III.

The Absorption of Agglutinin Contained in Homologous Serum Administered into the Trachea and Peritoneal Cavity.

Time blood was obtained.	Rabbit 8. Weight 2,350 gm. 6 cc. of serum from a rabbit immunized with the hog-cholera bacillus injected intratracheally.						Rabbit 9. Weight 2,670 gm. 6 cc. of serum from a rabbit immunized with the hog-cholera bacillus injected intraperitoneally.					
	Results of tests of rabbit serum for agglutinins specific for the hog-cholera bacillus.											
	Serum dilutions.						Serum dilutions.					
	1:10	1:20	1:50	1:100	1:200	1:500	1:10	1:20	1:50	1:100	1:200	1:500
Before injection.	−	−	−	−	−		++	+	±	−	−	−
After "												
hrs.												
½	−	−	−	−	−	−	C.	C.	++	+	±	−
1	−	−	−	−	−	−	"	"	++++	+	±	−
2	−	−	−	−	−	−	"	"	C.	++++	++	±
3	−	−	−	−	−	−	"	"	"	+++	++	+
4	+	−	−	−	−	−	"	"	"	+++	++	+
5	+	−	−	−	−	−	"	"	"	+++	++	+
6	+	±	−	−	−	−	"	"	"	+++	++	+
7	+	+	±	−	−	−	"	"	"	+++	++	+
24	++	+	+	−	−	−	"	"	"	++++	++	+

Little and Orcutt[5] have shown that the new-born calf for a short period after birth readily absorbs agglutinins for *Bacillus abortus* from the colostrum of the mother through the walls of the gastrointestinal canal. Smith and Little[6] fed young calves cow serum of high titer for *Bacillus abortus* and showed that the agglutinins were absorbed through the intestinal wall into the blood stream within a few hours. To test whether the epithelium of the alveoli and air tubes is more readily permeable to antibody in a homologous serum, the serum from a rabbit immunized with a strain of the hog-cholera bacillus was injected into the trachea of one rabbit and into the peritoneal cavity of another. This serum in a dilution of 1:10,000 would cause marked agglutination of the hog-cholera bacillus. As in the previous experiment, the rabbits were bled before and at regular intervals after the injection. The various samples of serum were tested for the presence of agglutinins with suspensions of the hog-cholera bacillus. The details are given in Table III.

The results of this experiment were similar to those obtained when foreign serum containing antibodies was injected into the trachea. The respiratory epithelium appears to be impermeable for antibodies contained in a homologous serum as it is for those contained in the serum of other species.

DISCUSSION AND SUMMARY.

The experimental results indicate that the walls of the lower respiratory tract are relatively as impermeable for antibodies as they are for serum proteins. The kind of serum employed does not influence this fact. Antibodies contained in homologous (rabbit) serum failed to enter the blood stream when injected into the trachea in greater concentration than those contained in heterologous (guinea pig and cow) sera.

That a little serum and its contained substances are absorbed after intratracheal injection has been shown both by the experiments dealing with sensitization and anaphylaxis previously reported and by the experiments recorded in this paper.

[5] Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.
[6] Smith, T., and Little, R. B., *J. Exp. Med.*, 1923, xxxvii, 671.

Besredka's assertion that the laryngotracheal method is an efficient one for the administration of serum in the treatment of disease has not been borne out by the experimental evidence here presented. From the results one would conclude that it is one of the least efficient routes for administration.

In contrast to the relative impermeability for proteins of the mucous membranes of the lower respiratory tract is the relative permeability of the endothelium of the peritoneal cavity. Antibody is there absorbed readily, whether it be in heterologous or in homologous serum. The indications are that a considerable portion of the antibody injected into the peritoneal cavity reaches the blood stream in 2 or 3 hours.

CAUSE OF IMMEDIATE DEATH BY LARGE DOSES OF BOTULINUS TOXIN.

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Ever since the discovery of the existence of soluble toxins in the culture filtrates of certain bacteria, it has been observed that these toxins differ radically from metallic or alkaloidal poisons in that they produce no immediate effect on experimental animals. The symptoms of intoxication by bacterial toxins appear only after a comparatively protracted period of latency. Within certain limits the duration of the period of latency in the action of toxins (so called period of incubation) was found to be in inverse relation to the amount of toxin administered. Thus, for instance, the symptoms of poisoning do not appear in experimental animals (mice) for several days after the injection of small amounts of tetanus toxin, but this period of latency can be shortened to 12 hours if 3,600 lethal doses of toxin are injected.[1] However, no matter how much toxin is injected in the case of tetanus or diphtheria toxin, the period of incubation cannot be reduced below 8 to 10 hours.

In the case of *botulinus* toxin, the period of incubation seems to be considerably shorter, but it may vary within very wide limits. In our own experience, among seven persons involved in a single outbreak which terminated in six deaths and one recovery, the time of the onset of symptoms varied from 4 to 54 hours after the ingestion of poisonous food.[2,3] When this toxin is introduced parenterally into experimental animals in small amounts, the first symptoms may

[1] De Waele, H., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 148.

[2] Sisco, D. L., *J. Am. Med. Assn.*, 1920, lxxiv, 516.

[3] Sisco, D. L., An epidemiological study of food poisoning, Thesis, Harvard University, 1920.

not appear before the expiration of 24 hours. When larger amounts of toxin are administered, one might expect a shortening of the incubation period comparable to that observed in the case of other bacterial toxins. However, in the course of study of toxicity of various food products experimentally infected with *Bacillus botulinus*, one of us (Orr) has observed that the injection of massive doses of such material (0.5 to 1 cc.) may cause death of animals in from 5 to 10 minutes after injection. Since this apparent absence of incubation period is inconsistent with the established conceptions concerning the mode of action of bacterial toxins, we thought it advisable to study the subject more closely.

A culture of *B. botulinus*, Type A, was grown at 37°C. for 5 days on minced meat glucose broth medium. At the end of this time, the liquid portion of the culture medium was decanted, forced through a sterile Berkefeld filter, diluted with physiological salt solution, and injected intraperitoneally into a series of mice in the amounts indicated in Table I. The animals were carefully observed and the character of the symptoms of intoxication, the time of their appearance, and the time of death noted. The experiment was repeated many times, with several batches of toxic culture filtrate. The results obtained varied somewhat with different filtrates and with different batches of mice. In the composite Table I we have entered two sets of results for each dose of toxin, the first figure indicating the shortest and the second the longest interval elapsing between the time of injection of toxin and death.[4]

As may be seen from these results, the interval of time elapsing between the intraperitoneal injection of the toxic filtrate and the death of mice is reducible from over 24 hours to about 1 hour by increasing the amount of toxin injected from a dose of 3×10^{-6} cc. to that of 3×10^{-2} cc. The animals receiving 3×10^{-2} cc. of toxin show at first no effects of injection and for about $\frac{1}{2}$ hour remain apparently normal, perhaps somewhat more quiet than control mice. Later, however, their respiration becomes progressively more labored, at first increasing in rate and then in depth. This change comes

[4] While it is customary to consider as the incubation period the time elapsing between the injection of the toxin and the appearance of the first unmistakable symptoms of intoxication, nevertheless in true *botulinus* poisoning in mice, the onset of symptoms is not very well defined and it varies considerably with individual animals. For this reason, we have recorded instead the time of death, which is more uniform.

on so gradually and is so mild at first that it is difficult to establish the exact time of its onset. In the next few minutes abdominal muscles become completely relaxed and flaccid; the apparent constriction at the diaphragm gives the animal a wasp-like appearance; it lies motionless on its abdomen and does not respond to external stimuli. The respiration rate falls very rapidly and may become as low as 5 per minute. The inspirations become extremely labored. The majority of animals present at this stage a marked exophthalmus. They soon go into coma which is not interrupted until death occurs, in about 1 hour from the time of the administration of toxin. But if the amount of the filtrate injected is much greater than 3×10^{-2} cc., the picture

TABLE I.

Period of Survival after Graded Doses of botulinus Toxin.

Amount of toxin injected, *cc.*...	10^{-0} 1	3×10^{-1} 0.3	3×10^{-2} 0.03	3×10^{-3} 0.003	3×10^{-4} 0.0003	3×10^{-5} 0.00003	3×10^{-6} 0.000003	3×10^{-7} 0.0000003
Minimum interval before death.....	5 min.	5 min.	1 hr.	1 hr.	2 hrs.	7 hrs., 45 min.	24 hrs.	Survived.
Maximum interval before death.....	7 "	1 hr.	1½ hrs.	2 hrs., 15 min.	6½ "	20 hrs.	55½ "	"

is strikingly different. The animals show definite restlessness and marked contraction of the abdominal muscles almost before the entire contents of the syringe have passed into the peritoneal cavity. Within a minute or two, they give an increased response to external stimuli, especially to sharp sound. Shortly they become prostrated, though the increased excitability persists a few minutes longer. Respiration increases in depth and decreases in frequency. The animal goes into coma interrupted by sharp convulsive seizures with clonic contraction of the extensor muscles throughout the body. Death occurs in 5 to 15 minutes after the injection and during one of these convulsive seizures. In several instances, particularly among the

larger mice receiving moderate amounts of toxin,[5] the animals exhibited most of the acute symptoms just described, but recovered temporarily, only to succumb later, that is to say within an hour or two, with symptoms of *botulinus* poisoning similar to those occurring in mice receiving smaller amounts of toxin.

Specificity of the Toxic Filtrate.

The observations just mentioned suggest the possibility that the filtrate contains two toxic substances,—one, acting slowly, apparently present in high concentration so that its presence can be demonstrated even in high dilutions of culture filtrate; the other causing acute immediate symptoms and detectable only when comparatively large amounts of filtrate are injected.

That *botulinus* toxin may have a composite nature seems quite possible, but,—by analogy with tetanus toxin, which is composed of tetanospasmin and tetanolysin,—it might be expected that if both the toxic components of *botulinus* filtrate are of the nature of true toxin, they should both be neutralized by the antitoxin. This was investigated in the following experiment.

Five test-tubes each received 0.2 cc. of the filtrate of a 5 day old culture of *B. botulinus*, Type A. This amount was selected, since the results of a preliminary experiment indicated it to be the largest amount of this culture filtrate which can be injected intraperitoneally in mice without causing acute immediate death. To each of these tubes containing toxin was introduced respectively 0.1, 0.05, 0.02, and 0.01 cc. of specific antitoxin, the fifth tube receiving no antitoxin (control). Similarly, another series of two test-tubes received each 0.5 cc. of the same culture filtrate,—an amount selected because it is certain to cause the symptoms of acute intoxication. To the contents of one of the latter tubes was added 0.5 cc. of antitoxin, that is to say at least four times as much as is necessary to neutralize the specific toxin present (Table II, Tube 2), while the other tube received no antitoxin (control). A third series of three tubes received respectively 0.3, 0.5, and 1 cc. of uninoculated sterile culture medium (minced meat glucose broth). The contents of each one of the ten tubes were brought to a uniform volume of 1 cc., by the addition of physiological salt solution, and incubated at 37°C. for 2 hours. At this time, the contents of the respective tubes were injected intraperitoneally into normal mice of 17 to 22 gm. and the results of this toxicity test were recorded in terms of time elapsing between the

[5] See second mouse receiving 0.3 cc. of toxin, Table I.

injection and death of animals. Three such experiments in all were performed. The time of death recorded in Table II represents respectively the minimum and the maximum periods of survival observed in the three experiments.

It will be seen that Guinea Pig 6, receiving a quantity of antitoxin sufficient to neutralize more than 1 cc. of specific toxin, died 5 minutes after injection of one-half this amount of the crude *botulinus* filtrate, exactly as did Guinea Pig 7 which received no antitoxin at all.

The results of this experiment indicate that the acute symptoms of intoxication observed after the injection of comparatively large

TABLE II.

Neutralizing Effect of Antitoxin on Large and Small Amounts of botulinus Toxin.

Tube No.		Amount of toxic culture filtrate.	Amount of sterile medium.	Amount of antitoxin.	Results.
		cc.	*cc.*	*cc.*	
	1	0.2		0.1	Survived.
	2	0.2		0.05	"
A.	3	0.2		0.02	Died in 36 to 56 hrs.
	4	0.2		0.01	" " 12½ " 20 "
	5	0.2 (control).		0.00	" " 1 hr. to 1 hr., 20 min.
B.	6	0.5		0.5	Died in 5 to 8 min.
	7	0.5 (control).		0.0	" " 5 " 10 "
	8		0.3	0.0	Survived.
C.	9		0.5	0.0	"
	10		1.0	0.0	"

doses of *botulinus* culture filtrate are due to the presence in this filtrate, in addition to a specific toxin, of some other poisonous substance which is not neutralized by an excess of specific antitoxin (Table II, *B*). Such a poisonous constituent must, nevertheless, be a product of the growth of *Bacillus botulinus* on minced meat glucose broth, since the original medium is free from this poison (Table II, *C*).

Nature of Toxic By-Product of botulinus Culture Filtrate.

In order to obtain some evidence as to the nature of the poison which causes acute intoxication in mice injected with large doses of *botulinus* filtrate, we attempted to determine its resistance to heat.

3 cc. of a filtrate of 5 day old culture of *B. botulinus* were heated for 5 minutes at 80°C. in a sealed test-tube. Such an exposure is known to be amply sufficient for the destruction of true toxin. The contents were quickly cooled by immersing the test-tube in cold water and injected intraperitoneally into a series of mice in the amounts indicated in Table III, *B*. Another portion of 3 cc. of the same filtrate was placed in an open graduated test-tube and subjected to boiling over a flame for 20 minutes. At the end of this time, the evaporated water was replaced to the original volume of 3 cc. and the contents of the tube injected into a series of mice as indicated in Table III, *C*. A third 3 cc. portion of the same filtrate was sealed in a glass tube and subjected to autoclaving for 20 minutes at 15 pounds pressure, after which the tube was cooled, opened, and the contents injected into mice as indicated in Table III, *D*. As a control, a fourth portion of filtrate was injected into mice without any preliminary treatment (Table III, *A*). The results of one such experiment are presented in Table III, in terms of the time elapsing between the injection and death of the mice.

TABLE III.

Effect of Heating in Open and Closed Containers on the Toxicity of B. botulinus Culture Filtrate.

	Amounts of filtrate injected.					
	1 cc.	0.7 cc.	0.5 cc.	0.3 cc.	0.2 cc.	0.00002 cc.
A. Untreated filtrate (control).....	6 min.	8 min.	10 min.	14 min.	1 hr., 10 min.	8 hrs.
B. Filtrate heated at 80°C., 5 min.....	5 "	5 "	10 "	10 "	Survived.	Survived.
C. Filtrate boiled 20 min. in open tube.	12 "	15 "	Survived.	Survived.	"	"
D. Filtrate autoclaved (sealed)....	5 "	5 "	6 min.	8 min.	"	"

These results show that the substance which is responsible for the toxicity of minute amounts of the filtrate; which acts only after an incubation period of not less than 1 hour (Table I); and which is capable of being neutralized by *botulinus* antitoxin (Table II, *A*) possesses still another property of true bacterial toxins—it is thermolabile (Table III, *B*).

As to the second toxic constituent of *botulinus* culture filtrate, active when it is given in large amounts and responsible then for immediate acute symptoms of poisoning and the rapid death of animals, it acts without any noteworthy incubation period (Table I),

is not neutralized by an excess of *botulinus* antitoxin (Table II, *B*), and is thermostable (Table III, *B*). The fact that this poison is weakened when boiled in an open vessel (Table III, *C*), while it retains its activity in a sealed container, even after autoclaving (Table III, *D*), may be taken to indicate that it is a chemical poison capable of volatilization.

Since *Bacillus botulinus* is known to possess marked proteolytic power, it was suspected that the immediate toxicity of the filtrates might be due to accumulation in the culture of some toxic products of putrefaction. In order to investigate this possibility, we have prepared filtrates from cultures of other putrefactive bacteria grown on minced meat glucose broth and tested their toxicity on mice. We found that the filtrates of cultures of *Bacillus proteus*, *Bacillus aerogenes*, and of atoxic strains of *Bacillus botulinus* were all able to elicit in mice symptoms of acute intoxication identical with those already described. Moreover, the substances responsible for the toxicity of these filtrates were also found to be relatively thermostable and volatile.

Some time ago, while studying the nature of "toxin" produced by *Bacillus œdematis maligni*, Barger and Dale[6] concluded that the toxicity of the filtrates of this organism is due to accumulation of ammonium salts. In order to ascertain whether the toxic substances accumulating in the cultures of *Bacillus botulinus* could be ammonium salts, a small portion of the filtrate was made alkaline by the addition of an excess of NaOH solution, subjected to boiling, and the reaction of the vapor tested with litmus paper. This test showed that the filtrate contained an appreciable amount of volatile bases. When the filtrate was subjected to aeration by the method of Folin, it was found that a considerable portion of the volatile bases could be identified as ammonia. The aeration of various samples showed that 1 cc. of the filtrate may contain from 0.01 to 0.05 gm. of ammonia, depending upon the age of the culture.

In order to determine whether this amount of ammonia could alone be responsible for the acute intoxication of animals, we injected mice intraperitoneally with solutions of ammonium chloride and

[6] Barger, G., and Dale, H. H., *Brit. Med. J.*, 1915, ii, 808.

ammonium sulfate, the ammonium content of which was nearly equivalent to that present in the filtrate. The results of this experiment are shown in Table IV, in terms of period of survival after injection.

The results of this experiment indicate that the toxicity of ammonium salts is comparable to that of *Bacillus botulinus* filtrates when the doses are selected so as to contain approximately equivalent amounts of ammonia. Indeed this similarity and the similarity of symptoms elicited are so striking that one may be allowed to conclude that the acute toxicity of the large doses of culture filtrates of *Bacillus botulinus* is due directly to the ammonium salts present in them.

TABLE IV.

Ammonium chloride.			Ammonium sulfate.			*Botulinus* toxin.		
Amount of salt injected.	Amount of NH_3 calculated.	Result of injection.	Amount of salt injected.	Amount of NH_3 calculated.	Result of injection.	Amount of toxin injected.	Amount of NH_3 calculated.	Result of injection.
gm.	*gm.*		*gm.*	*gm.*		*cc.*	*gm.*	
0.1	0.032	2½ min.	0.1	0.025	4 min.	1	0.010	5 min.
0.05	0.016	4 "	0.05	0.012	5 "	0.5	0.005	7 "
0.025	0.008	6 "	0.025	0.006	6 "	0.25	0.002	65 "
0.01	0.003	50 "	0.01	0.002	Recovered.	0.1	0.001	55 "
0.005	0.001	Recovered.	0.005	0.001	"			

SUMMARY AND DISCUSSION.

Parenteral introduction of amounts of the culture filtrate of *Bacillus botulinus* greatly in excess of the minimum lethal dose has been observed to cause the practically immediate death of mice. This result is due to the presence in the filtrates of a chemical poison possessing properties distinct from those of the contained *botulinus* toxin which itself acts only after a well defined period of incubation. This chemical poison is not neutralized by *botulinus* antitoxin; it is effective only when large amounts of the culture filtrate are given; it is thermostable, not being destroyed when heated in the autoclave in a sealed tube, though when it is heated in an open container its toxicity diminishes with a coincidental volatilization of basic material. The volatile substance can be identified as ammonia.

Death resulting from the injection of comparatively large amounts of ammonium salts (0.1 gm.) is easily distinguished from that due to botulism, both through the character of the symptoms and the absence of an incubation period. However, when the amount of toxic salts injected is smaller (0.01 gm.), the symptoms of poisoning are not so characteristic and death may be delayed long enough to suggest a period of incubation similar to that observed in botulism (Table IV). This circumstance is of importance in connection with the examination of partly decomposed food products in which the presence of *botulinus* toxin is suspected. As a rule such suspected material is injected in massive doses (0.5 to 1 cc.) in mice.[7,8] It is conceivable that such spoiled foods may be contaminated with common putrefactive bacteria yielding ammonia during their growth and thus may cause death of the test animals. If in such tests mice passively protected by the preliminary injection of an excess of antitoxin be used in addition to normal animals, the chances of an error in the interpretation of the results will be materially reduced, though not ruled out. Unfortunately for such a procedure, *botulinus* antitoxin is not readily available, while furthermore, recent findings[9] indicate that it may not always be effective owing to the existence of a group of toxin-producing bacteria very similar to *Bacillus botulinus*, but not homologous immunologically with either of the known types of the latter.

The test of thermostability of the toxic constituents of suspected food may conceivably help to determine the true nature of the poison.

[7] Orr, P. F., *J. Infect. Dis.*, 1921, xxix, 287.
[8] Bengtson, I. A., *Pub. Health Rep., U. S. P. H.*, 1921, xxxvi, 1665.
[9] Bengtson, I. A., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 340.

ON THE SPECIFICITY OF AGGLUTININS AND PRECIPITINS.*

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The knowledge of serological reactions has opened a field of research dealing with the specific configurations existing in the chemical constituents of organisms. The results of agglutinin and lysin reactions and the use of the absorption method have disclosed an unexpected variety of structures as exemplified by the existence of differently reacting groups in a single kind of cells, of differences between individuals of the same species, of various types among bacilli of the same sort, and so on.

The question whether the methods of absorption or partial saturation applied to precipitin reactions will disclose analogous facts is discussed in the present paper. Should this be the case, it might be possible to isolate antibodies which could serve to discriminate chemical groups contained in the protein molecules involved in precipitin reactions. For present purposes the nature of the precipitins reacting strongly on antigens of remote species will be left out of account because in their case special factors are possibly concerned. These reactions have recently been studied by Friedberger and his coworkers[1] and have been termed by them "heterogenetic precipitations."

Experiments on Partial Saturation of Precipitins.

The experiments on partial saturation of precipitins reported by previous writers include instances of two sorts. In the one an

* Nineteenth paper on antigens and specificity.

[1] Friedberger, E., and coworkers, *Z. Immunitätsforsch., Orig.*, 1919, xxviii, 237; 1920, xxx, 351; 1923, xxxvi, 233, 272, 386; 1923–24, xxxviii, 184, 264; *Biochem. Z.*, 1923, cxxxvii, 312; *Klin. Woch.*, 1922, i, 1248. *Cf.* Manteufel, P., and Beger, H., *Z. Immunitätsforsch., Orig.*, 1921–22, xxxiii, 348. Yu, I., *Centr. Bakt., 1. Abt., Orig.*, 1923, xc, 381. Wells, H. G., Meeting of the American Association of Immunologists, Boston, 1923.

antiserum is obtained through the injection of an antigen composed of several serologically different proteins. The several antibodies which it in consequence contains are thrown down separately by the consecutive addition of the individual antigenic components. Experiments of this kind have been performed by Ascoli[2] Ide,[3] von Dungern,[3] and others with the proteins of serum—globulin, albumin—which, as it happens, behave serologically like entirely different substances.[4] In instances of the second sort, a precipitin is produced by the injection of a single protein or of a mixture of proteins of the same animal derivation. These precipitins react, though to a lesser degree, upon the proteins of related species. In such cases it is possible to assume that the precipitation occurring with material from related species comes about as a result of a similarity in the chemical composition of the strange material and that used as antigen. Several authors have investigated the problem thus raised by means of experiments involving the partial saturation of precipitins.[5] The conclusion is generally drawn, in conformity with the experiments of Ehrlich and Morgenroth,[6] that the reactions of a single immune serum with several antigens are referable to a multiplicity of antibodies corresponding to various chemical groups in the homologous antigen, some of these groups being present also in the related antigens and thus giving rise to the overlapping reactions.

The method of partial saturation of precipitins has been but little used for biological and even practical purposes.[7] While this is possibly due to the technical difficulty of determining the proper conditions of

[2] Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 1409.

[3] Ide, M., *Bull. Acad. Roy. méd. Belg.*, 1903, xvii, series 4, 913. von Dungern, E. F., *Centr. Bakt., 1. Abt., Orig.*, 1903, xxxiv, 355; Die Antikörper, Jena, 1903.

[4] Dale, H. H., and Hartley, P., *Biochem. J.*, 1916, x, 408. Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1922, xcvi, 191.

[5] Weichardt, W., *Hyg. Rundschau*, 1903, xiii,756; *Z. Med.-Beamte*, 1902, xv, 729. Ide.[3] von Dungern.[3] Manteufel, T., and Beger, H., *Z. Immunitätsforsch., Orig.*, 1921–22, xxxiii, 367, 370. Welsh, D. A., and Chapman, H. G., *J. Hyg.*, 1910, x, 177. Ascoli.[2]

[6] Ehrlich, P., and Morgenroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 569, 598.

[7] *Cf.* Uhlenhuth, P., *Beihefte med. Klin.*, 1907, iii, 246, 252.

the experiments,[8] the principle of the method is likewise open to doubt. For after removing the precipitate formed by the addition of an heterologous antigen, a quantity of this latter may still be present in the fluid, side by side with, or perhaps in a soluble combination with, residual antibodies.[9] It is conceivable that such a mixture may not react upon a further addition of heterologous antigen and yet will do so with an homologous antigen endowed with higher affinity. As a matter of fact, the supernatant fluid remaining after partial precipitation with an homologous antigen reacts with this same substance, if it be added in a higher concentration than that used for the first precipitation, as Coca and Kosakai[10] have shown by experiments with the pseudoglobulin of serum and crystalline egg albumin. From similar experiments which we ourselves have made, it will be seen that the solutions, after partial neutralization, did not only behave like higher dilutions of antiserum, but actually showed a relatively rapid decrease in the intensity of the reaction when the concentration of antigen was diminished (Table 1).

3 cc. of horse serum, diluted 1:100, were mixed with 0.6 cc. of rabbit anti-horse precipitin, kept for 2 hours at room temperature and overnight in the ice box, and then centrifuged. 0.5 cc. of the supernatant fluid was taken for every test and 0.1 cc. of various dilutions of horse serum added. In the same way dilutions of untreated immune serum were tested. Readings were made after 3 hours standing at room temperature and overnight in the refrigerator. The strength of the reactions is indicated as follows: F. tr. = faint trace; Tr. = trace; ±, +, +±, etc.

All the immune sera employed were obtained from rabbits immunized in the usual way by weekly intraperitoneal or intravenous injections.

Like results were obtained in similar tests with an anti-human precipitin.

The following experiments show the effects of partial saturation with, and subsequent addition of, homologous and heterologous antigen.

[8] Fürth, J., *Arch. Hyg.*, 1923, xcii, 158.

[9] *Cf.* Zinsser, H., and Young, S. W., *J. Exp. Med.*, 1913, xvii, 396. Michaelis, L., and Fleischmann, P., *Z. exp. Path. u. Therap.*, 1905, i, 547. Opie, E. L., *J. Immunol.*, 1923, viii, 19. Nachtergael, A., *La Cellule*, 1905, xxii, 125.

[10] Coca, A. F., and Kosakai, M., *J. Immunol.*, 1920, v, 297.

To a series of tubes containing 1.5 cc. of various dilutions of human and monkey serum, 0.3 cc. anti-human precipitin was added. After 2 hours at room temperature and overnight in the ice box, the tubes were well centrifuged in order to remove the precipitates formed. After centrifugation some of the tubes still showed slight turbidity, which was allowed for in the readings.

To 0.4 cc. of the supernatant fluid, 0.2 cc. of a 1:500 dilution of human and monkey serum (*Macacus rhesus*) was added (Table II, *a*). In another series of tubes, there was added instead the antigen used for the previous precipitation in a concentration five times greater than before (Table II, *b*). Readings were made after 2 hours standing at room temperature.

TABLE I.

Horse serum diluted.	Supernatant fluid.	Untreated immune serum diluted.				
		1:6	1:10	1:20	1:50	1:100
1:50	+	+++	+++	+±	±	Tr.
1:100	±	++	+++	++	±	F. tr.
1:250	Tr.	+±	++	++±	±	" "
1:500	0	+	+±	+±	±	" "
1:1,000	0	+	+	+	±	" "

TABLE II, *a*.

Supernatant fluid after precipitation with human serum diluted.	Human serum 1:500.	Monkey serum 1:500.	Supernatant fluid after precipitation with monkey serum diluted.	Human serum 1:500.	Monkey serum 1:500.
1:1,000	+±	+±	1:1,000	+±	Tr.
1:300	+	Tr.	1:300	+±	"
1:100	Tr.	0	1:100	+±	F. tr.
1:30	0	0	1:30	+	0
1:15	0	0	1:15	+	0

It will be seen from the experiment that anti-human precipitin saturated with monkey serum reacts specifically on human protein. Evidently, therefore, the serological differentiation between monkey and human serum is not particularly difficult, as Fujiwara has recently shown anew.[11] However, by using higher concentrations of the antigen, the fact is demonstrable that the liquid resulting from the partial saturation still contains antibodies reacting on monkey serum.

[11] Fujiwara, K., *Z. ges. gerichtl. Med.*, 1922, i, 754.

A similar experiment was made with anti-chicken precipitin, chicken, and goose serum, the quantitative relations being the same (Table III, *a*). Additional tests in which various concentrations of homologous antigen were added to the supernatant fluid are summarized in Table III, *b*. Readings were made after 4 hours at room temperature (*cf.* Table IX).

TABLE II, *b*.

Supernatant fluid after precipitation with human serum diluted.	Human serum.*	Supernatant fluid after precipitation with monkey serum diluted.	Monkey serum.*
1:1,000	++	1:1,000	Tr.
1:300	+	1:300	+
1:100	+	1:100	+
1:30	+±	1:30	+±
1:15	Tr.	1:15	Tr.

* Concentration five times greater than that indicated in Column 1.

TABLE II, *c*.

0.4 cc. untreated immune serum diluted.	0.2 cc. human serum 1:500.
1:6	++
1:12	+±
1:24	+
1:48	Tr.
1:96	F. tr.

TABLE II, *d*.

	1.5 cc. human serum diluted.					1.5 cc. monkey serum diluted.				
	1:15	1:30	1:100	1:300	1:1,000	1:15	1:30	1:100	1:300	1:1,000
0.3 cc. immune serum	++	++	+++	++	+	++	+±	+	+±	+

In order to avoid, to a certain extent, the complications arising from the presence of several proteins in serum (*cf.* the results by Opie[9]) an experiment similar to the foregoing one was made with a precipitin for horse euglobulin. For the immunization, the fraction precipitated at a 33 per cent saturation with ammonium sulfate was used. The immune serum proved to be highly specific when solutions of euglobulin and albumin—precipitated between 55 and 100

per cent saturation with ammonium sulfate—were tested. With the latter substance no reaction took place up to concentrations of 1 to 2 per cent.

TABLE III, *a*.

Supernatant fluid after precipitation with chicken serum diluted.	Chicken serum 1:500.	Goose serum 1:500.	Supernatant fluid after precipitation with goose serum diluted.	Chicken serum 1:500.	Goose serum 1:500.
1:1,000	+±	+±	1:1,000	+±	+
1:300	+	±	1:300	+	0
1:100	F. tr.	0	1:100	±	0
1:30	0	0	1:30	Tr.	0
1:15	0	0	1:15	"	0

TABLE III, *b*.

Supernatant fluid after precipitation with chicken serum diluted.	Chicken serum.					Supernatant fluid after precipitation with goose serum diluted.	Goose serum.				
	1:200	1:60	1:20	1:6	1:3		1:200	1:60	1:20	1:6	1:3
1:1,000	++	+++	++	+	+±	1:1,000	+	+	+	++	+±
1:300	+±	+	+	+	+±	1:300	Tr.	±	+	+±	±
1:100	Tr.	±	+	+	+±	1:100	0	Tr.	+	+	±
1:30	0	Tr.	±	±	±	1:30	0	0	Tr.	0	Tr.
1:15	0	0	0	0	0	1:15	0	0	0	0	0

TABLE III, *c*.

0.4 cc. untreated immune serum diluted.	0.2 cc. chicken serum 1:500.	0.2 cc. goose serum 1:500.
1:6	++	++
1:12	+±	+
1:24	+	±
1:48	±	F. tr.
1:96	Tr.	0
1:192	0	0

The results (Table IV) are like those obtained with precipitins against whole serum. However, in other experiments with two sera containing antibodies effective against horse serum albumin, the phenomenon of apparent specificity after partial neutralization with donkey serum was hardly perceptible.

A peculiarity noted in the experiment with horse and chicken immune serum (Tables III and IV) is the decrease in intensity of the specific reaction when the amount of heterologous antigen taken for the partial saturation is increased.

When antigens unrelated serologically are tested, the results are different (Table V).

In such an experiment the strength of the reaction was not influenced by the quantity of heterologous antigen employed in the

TABLE IV, *a*.

Readings made after 2 hours.

Supernatant fluid after precipitation with horse serum diluted.	Horse serum 1:500.	Donkey serum 1:500.	Supernatant fluid after precipitation with donkey serum diluted.	Horse serum 1:500.	Donkey serum 1:500.
1:1,000	+	±	1:1,000	+	Tr.
1:300	Tr.	F. tr.	1:300	+	F. tr.
1:100	0	0	1:100	±	0
1:30	0	0	1:30	0	0
1:15	0	0	1:15	0	0

TABLE IV, *b*.

Supernatant fluid after precipitation with horse serum diluted.	Horse serum.*	Supernatant fluid after precipitation with donkey serum diluted.	Donkey serum.*
1:1,000	+±	1:1,000	+
1:300	++	1:300	+
1:100	+	1:100	+
1:30	±	1:30	±
1:15	Tr.	1:15	Tr.

* Concentration five times greater than that indicated in Column 1.

initial precipitation. In cases in which a decrease is observed, one may ascribe it either to a reaction of the heterologous antigen with almost the whole amount of antibodies in forming a precipitate or to inhibition of the precipitation by an excess of antigen.[12] This latter phenomenon, which is probably due to

[12] Camus, M. L., *Compt. rend. Acad.*, 1901, cxxxii, 215. Halban, J., and Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 473. Michaelis, L., *Beitr. chem. Physiol. u. Path.*, 1904, iv, 59. Eisenberg, F., *Bull. internat. Acad. sc. Cracovie*, 1902, 289.

the formation of a soluble compound between antibody and antigen, is also indicative of a specific affinity. The phenomenon of inhibition is not equally well pronounced with every precipitin serum. But if the serum is appropriate, even those related heterologous antigens which give only a weak precipitation as compared with the homologous antigen have the power to inhibit the reaction with the homologous antigen to a high degree. An example is furnished by tests with a precipitin for rat serum which gave also a weak reaction with mouse serum (Table VI). A number of normal sera were employed for comparison.

TABLE V.

The arrangement of the experiment was like that of the previous ones. Immune serum against whole horse serum was employed, and for the neutralization and the tests, solutions of euglobulin and albumin of horse serum. The undiluted solution contained about 7.5 per cent protein. Readings were made after 2 hours at room temperature and overnight in the ice box.

Supernatant fluid after precipitation with albumin solution diluted.	Euglobulin solution 1:500.	Albumin solution 1:500.
1:1,000	++	0
1:300	++	0
1:100	++	0
1:30	++	0
1:15	++	0
Untreated immune serum.		
1:6	++	++

It would appear from Table VI that despite the wide difference in strength exhibited in the direct reaction, mouse serum inhibits almost as well as rat serum. The weaker but not insignificant inhibition produced by some sera of Ungulata may be ascribed, perhaps properly, to a certain affinity of the antibodies for these proteins and in that respect it is worth noting that no alteration at all was brought about by the sera of birds.

We have tried to effect the absorption of precipitins by a method similar to that used for agglutinins[13] and have employed for this purpose proteins coagulated by heat or alcohol. The results were not wholly satisfactory owing to the difficulty of obtaining clear solutions after centrifugation, as also because of the

[13] *Cf.* Friedberger, E., and Meissner, G., *Z. Immunitätsforsch., Orig.*, 1923, xxxvi, 233

large amounts of coagulated material necessary for complete absorption, and because small amounts of the proteins went into solution, as shown by addition of precipitin. The outcome of the tests was similar to that obtained by the method of partial precipitation with soluble proteins. The amount of apparently specific antibodies left behind after a treatment with coagulated heterologous protein seemed to be considerably smaller than in the analogous tests with hemagglutinins, material from the same species being employed in both cases (see Table VIII). In judging the results of such experiments, the fact has to be taken into account that a decrease in the quantity of antibodies often affects the strength of the heterologous reaction more than that of the homologous one.

TABLE VI, *a*.

0.1 cc. of precipitin for rat serum. 0.2 cc. of dilutions of rat, mouse, and guinea pig serum. Readings made after 1 hour at room temperature.

Rat serum diluted.						Mouse serum diluted.						Guinea pig serum diluted.					
1:20	1:50	1:150	1:500	1:1,000	1:2,000	1:20	1:50	1:150	1:500	1:1,000	1:2,000	1:20	1:50	1:150	1:500	1:1,000	1:2,000
+++	++++	+++	++±	+	Tr.	±	+	±	±	Tr.	F. tr.	0	0	0	0	0	0

TABLE VI, *b*.

To 0.1 cc. of precipitin for rat serum were added 0.2 cc. of various normal sera (to the control tube 0.2 cc. of saline) and 10 minutes later 0.1 cc. of rat serum diluted 1:125. Readings made after 1 hour.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Control.
+±	+±	+±	++	F. tr.	Tr.	++	+	+±	+	++	+	+	+±	++	++	++	++

1, human; 2, monkey; 3, cat; 4, rabbit; 5, rat; 6, mouse; 7, guinea pig; 8, pig; 9, goat; 10, sheep; 11, beef; 12, horse; 13, donkey; 14, mule; 15, chicken; 16, goose; 17, pigeon.

Partial Saturation of Precipitins against Azoproteins.—As a further means of investigating the problem under discussion, we have made use of precipitins for antigens containing specific groups of known chemical constitution.[14] Sera were prepared by injecting rabbits with an azo compound formed by coupling horse serum with diazotized meta-amino-benzene-sulfonic (metanilic) acid. For *in vitro* tests

[14] Landsteiner, K., and Lampl, H., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 258, 293; *Biochem. Z.*, 1918, lxxxvi, 343; 1919, xciii, 106; 1920, civ, 280.

azo compounds prepared from chicken serum were employed.[14] As established previously, such sera react strongly with the homologous (metanilic acid) compound but also to a lesser degree on some other azoproteins with chemically similar azo components. For the heterologous reaction, the azo compound formed by coupling chicken serum with *p*-chlor-*o*-amino-benzene-sulfonic acid $C_6H_3(NH_2)^1(SO_3H)^2Cl^4$.

TABLE VII, *a*.

Technique as already given; readings made after 4 hours at room temperature; azoprotein from metanilic acid = M, from *p*-chlor-*o*-amino-benzene-sulfonic acid = C. The solution of azoprotein contained about 5 per cent protein.

Supernatant fluid after precipitation with M diluted.	Solution M 1:500.	Solution C 1:500.	Supernatant fluid after precipitation with C diluted.	Solution M 1:500.	Solution C 1:500.
1:1,000	0	0	1:1,000	+	Tr.
1:300	0	0	1:300	+	0
1:100	0	0	1:100	±	0
1:30	0	0	1:30	±	0
1:15	0	0	1:15	Tr.	0

TABLE VII, *b*.

To 0.2 cc. of various dilutions of Solutions M and C was added 0.04 cc. of precipitin for M. Readings were made after 2 hours at room temperature.

Solution M diluted.						Solution C diluted.					
1:15	1:30	1:100	1:300	1:1,000	1:3,000	1:15	1:30	1:100	1:300	1:1,000	1:3,000
±	±	+±	++	++	+	0	Tr.	+	+	+	±

was taken. As shown in Table VII, the immune serum gave a precipitation with this substance distinctly weaker than that with the homologous antigen, in analogy with the group reactions of natural antigens.

Since the chemical constitution of the specific groups of the two substances mentioned is fully known, a valid conclusion can be drawn. From a chemical point of view it is impossible to believe that two kinds of antibodies developed in the serum of the immunized animal, one of them acting on a common group of both antigens, the other on a

special group of the homologous material. On the other hand, a reaction of one antibody on the two chemically similar groups is quite conceivable, and the conception is supported by previous work.[14] The fact remains that after partial saturation with the homologous substance, the same occurrence took place as in the similar experiments with natural antigens; namely, a specific reaction of the supernatant fluid with the homologous, more active antigen. Similar results could undoubtedly be obtained with other azoproteins.

Experiments on Hemagglutinins.

The following tests were performed in order to compare the specificity and partial absorption of hemagglutinins and precipitins. Experiments of the sort with immune agglutinins and lysins have been carried out by several authors,[15] following the well known work of Ehrlich and Morgenroth.

The titration was made by mixing 0.5 cc. of various dilutions of rabbit immune agglutinins inactivated in the usual way with 0.05 of a 2.5 per cent emulsion of washed blood (0.1 cc. in the case of the emulsion of bird blood). The readings were made after 2 hours standing at room temperature. The figures given in Table VIII indicate the highest dilutions at which agglutination could still be discerned microscopically.

For absorption one-half volume of well centrifuged blood sediment from various species of animals was added to the immune serum diluted 1:10 to 1:40. The mixture was kept for 2 hours at room temperature and overnight in the ice box. Hemolytic tests were made only occasionally. They seemed to parallel the results of the agglutination tests.

It is evident from Table VIII that the titer of the hemagglutinins contained in the immune rabbit serum may be quite different for related species, as is shown, for instance, in the tests with human and monkey red cells. The lack of parallelism between these findings and those with precipitins is seen still more strikingly in the tests with anti-chicken immune serum.

[15] von Dungern, E., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 531. Landsteiner, K., and Reich, M., *Z. Hyg. u. Infectionskrankh.*, 1907–08, lviii, 213. Morgenroth, J., and Bieling, R., *Biochem, Z.*, 1922, cxxxi, 525.

The high titer of the chicken agglutinins for goose blood is probably referable to the normal rather than to immune agglutinins since similar titers have been noted with normal rabbit serum. Moreover, these agglutinins were not absorbed by chicken blood.

TABLE VIII.

	Blood.		
	Human.	Monkey.	
Agglutinin for human blood I	1:3,000	1:240	
" " " " I 1/20 absorbed with human blood	1:80	1:40	
Agglutinin for human blood I 1/20 absorbed with monkey blood	1:2,000	1:<20	
Agglutinin for human blood II	1:4,000	1:640	
" " " " II 1/20 absorbed with monkey blood	1:3,000	1:<20	
Agglutinin for human blood III	1:1,000	1:100	
	Guinea pig.	Mouse.	Rat.
Agglutinin for guinea pig blood I	1:1,000	1:160*	1:60*
" " " " " II	1:1,000	1:160*	1:80*
" " rat blood		1:3,200	1:6,400
" " " " 1/20 absorbed with rat blood		1:20	1:20
Agglutinin for rat blood 1/20 absorbed with mouse blood		1:20	1:3,200
	Horse.	Donkey.	
Agglutinin for horse blood I	1:10,240	1:2,560	
" " " " I 1/10 absorbed with horse blood	1:10	1:10	
Agglutinin for horse blood I 1/10 absorbed with donkey blood	1:2,560	1:20	
Agglutinin for donkey blood I	1:1,600	1:3,200	
" " " " I 1/10 absorbed with horse blood	1:10	1:800	
Agglutinin for donkey blood I 1/10 absorbed with donkey blood	1:160	1:<10	
Agglutinin for donkey blood II	1:8,000	1:12,000	
" " " " II 1/40 absorbed with horse blood	1:40	1:6,400	
Agglutinin for donkey blood II 1/40 absorbed with donkey blood	1:60	1:40	

* Similar titers were found with normal rabbit serum.

TABLE VIII—*Concluded.*

	Blood.			
	Chicken.	Guinea hen.	Goose.	Pigeon.
Agglutinin for chicken blood I..	1:800	1:300	1:1,600*	1:40
" " " " I 1/10 absorbed with chicken blood..	1:<20	1:80	1:1,600	1:40
Agglutinin for chicken blood I 1/10 absorbed with guinea hen blood..	1:640	1:<20	1:1,600	1:40
Agglutinin for chicken blood I 1/10 absorbed with goose blood..	1:800	1:200	1:40	1:40
Agglutinin for chicken blood I 1/10 absorbed with pigeon blood..	1:800	1:400	1:1,600	1:<20
Agglutinin for chicken blood II..	1:1,200	1:200	1:400*	1:100
" " " " II 1/10 absorbed with chicken blood..	1:<10	1:20	1:400	1:10
Agglutinin for chicken blood II 1/10 absorbed with guinea hen blood..	1:800	1:<10	1:400	1:10
Agglutinin for chicken blood II 1/10 absorbed with goose blood..	1:1,600	1:200	1:10	1:160
Agglutinin for chicken blood III..	1:800	1:400	1:800*	1:80
" " " " III 1/10 absorbed with chicken blood..	1:<10	1:20	1:800	1:10
Agglutinin for chicken blood III 1/10 absorbed with guinea hen blood..	1:800	1:<10	1:800	1:10
Agglutinin for chicken blood III 1/10 absorbed with goose blood..	1:1,600	1:400	1:20	1:120

For comparison with the agglutinin tests, an experiment on the specificity of chicken precipitin is presented in Table IX.

The low degree of specificity instanced by this case has been observed before by Uhlenhuth[16] among others, and Higashi[17] has had similar findings in the precipitation of bird hemoglobin. As a result of such observations, the idea has been put forward that species specificity as shown by serological tests is less pronounced in birds than in mammals. But we incline to think the phenomenon due to the wide distance of the bird from the animal used for obtaining the immune serum; that is to say, the rabbit. In keeping with this idea is the higher degree of specificity of the precipitin reactions obtained with immune sera derived from rabbits, within the order of Rodentia, as already established by previous work[18] (see Table VI, *a*).

[16] Uhlenhuth, P., *Deutsch. med. Woch.*, 1905, xxxi, 1673.

[17] Higashi, S., *J. Biochem.*, 1923, ii, 315.

[18] Trommsdorff, R., *Arb. k. Gsndhtsamte*, 1909, xxxii, 560. Graetz, F., *Z. Immunitätsforsch., Orig.*, 1910, vi, 627; *cf.* Steffenhagen, K., and Schoenburg, *Z. Immunitatsforsch., Orig.*, 1910–11, viii, 563.

In all cases, after treatment of the immune serum with heterologous blood, a fraction was obtained which was highly active and specific for the homologous blood, even when such closely related species as the horse and donkey were tested. In this case the original titer may have been nearly or quite the same for both kinds of blood. The result was not essentially different after a second similar treatment with the heterologous blood. These findings indicate that the differentiation of blood of closely related species is often easier by means of agglutinins than by precipitin reactions. Agglutinins can very probably be employed when precipitation tests meet with difficulty, for instance in the demonstration of the difference between the blood of human beings and of anthropoids.

TABLE IX.

0.2 cc. of various dilutions of normal sera was added to 0.05 cc. of precipitin for chicken serum. Readings were made after 1 hour at room temperature.

Chicken serum diluted.						Guinea hen serum diluted.					
1:10	1:50	1:150	1:500	1:1,500	1:5,000	1:10	1:50	1:150	1:500	1:1,500	1:5,000
+±	++	+++	+±	+	±	+±	++	++	+	±	Tr.
Goose serum diluted.						**Pigeon serum diluted.**					
1:10	1:50	1:150	1:500	1:1,500	1:5,000	1:10	1:50	1:150	1:500	1:1,500	1:5,000
+±	+±	++	+±	±	Tr.	+	++	+±	+	±	0

The corpuscles of varieties or races within a given species showed much less difference, if any, as compared with those manifested by corpuscles of closely related species. Experiments under way would seem to indicate that the method may be usefully employed for estimating the degree of relationship of similar animals.

A source of error which has not been taken into account by some previous workers is to be found in the existence of differences in the blood corpuscles of individuals of the same species. A high serum titer for the blood of one individual may persist after absorption with the cells of another individual.[19] Errors due to such cause can only be excluded by the examination of a number of different specimens.

[19] Landsteiner, K., and van der Scheer, J., *J. Immunol.*, 1924, ix, 213, 221.

DISCUSSION.

The conception, based on the findings of Ehrlich and Morgenroth with agglutinins, that precipitins contain in general a multiplicity of antibodies, each acting on a single chemical group of the antigenic proteins, is not conclusively proven. Some authors[3,20] have even assumed that the antigenic proteins also have a complex composition, as that, for instance, the serum globulin of one animal will contain fractions specific for that species and certain others present in different species as well. This view as a general principle is contradicted by the known facts. Were it true, a hemoglobin of one source should crystallize in many different forms since hemoglobin behaves serologically like other proteins.[21,17] But observation shows that this is not the case. On the other hand, the conception of a multiplicity of antibodies in the sense indicated above also meets with some difficulties, as reflection will show. One has to account not only for the differing behavior of a given immune serum against two antigens, but for the fact that precipitins in general show a maximum activity with the homologous protein, the reaction decreasing gradually in strength with the distance in the zoological scale[22] when other antigens are tested.

The results of precipitin reactions with antigen containing binding groups of known chemical constitution leave no doubt as to the fact that a single precipitin will regularly react with other substances if their chemical structure is sufficiently near to that of the homologous antigen.[14] The alternative idea, so widely accepted,—that a given immune serum which reacts on the homologous antigen A and also on antigen B derived from another species consists of antibodies specific for a common group of A and B and contains other antibodies specific for a group peculiar to A,— is not tenable in this case. It is reasonable, therefore, to assume that the affinity of antibodies for natural antigens is not absolutely restricted to a single substance, but can extend to other similar ones.

[20] Arrhenius, S., Immunochemie, Leipsic, 1907.

[21] See Landsteiner, K., *Kon. Akad. Wetensch. Amsterdam*, 1921, xxix, 1029; Heidelberger, M., and Landsteiner, K., *J. Exp. Med.*, 1923, xxxviii, 561.

[22] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

Since the antibodies which develop in various animals after injection of antigens, or in one individual at different stages of immunization, vary with regard to affinity and specificity, it is probable that in a single immune serum, as well, several such fractions are present and may possibly be separated. But one may suppose that each of them has a definite range of affinity for diverse antigens and that the phenomena observed in partial saturation do not fundamentally depend on a multiplicity of antibodies.

In the hemagglutinin experiments, a considerable part of the antibodies combined only with the homologous antigen, even when two closely related species were examined. The question arises whether these antigens are simple species-specific proteins or whether other factors are concerned in their specificity. They may possibly consist of such proteins combined with other substances or groups whose structure, unlike that of the proteins themselves, is not in close correspondence with the zoological system.[23] Instances which support this view are to be found in Forssman's heterogenetic antigens and in the isoagglutinin groups of human beings and animals. The fact that hemagglutinins of normal sera react frequently upon the corpuscles of related species as strongly as upon those of distant ones points in the same direction. Furthermore, the existence of similar groups in the blood corpuscles of distant species has been demonstrated in a general way by the method of splitting the combination of agglutinin and blood.[24] When, for instance, rabbit blood is agglutinated by beef serum, the combination can be dissociated by heating to 40–50°C., and the resulting liquid agglutinates not only rabbit corpuscles but also goose and frog blood markedly.

If, on the other hand, the hemagglutinogens should prove to consist only of proteins, they may have structural peculiarities, since proteins generally show a continuous variation by slow degrees

[23] Mention should be made of the possibly analogous relations described by Avery and Heidelberger in their immunological studies of the cell constituents of pneumococci.

[24] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905. *Cf.* Landsteiner, K., in Oppenheimer, C., Handbuch der Biochemie, Jena, 1910, ii, pt. 1, 410. Landsteiner, K., and Reich, M., *Z. Hyg. u. Infectionskrankh.*, 1907,–08, lviii, 213. *Cf.* Bailey, C.E., *Am. J. Hyg.*, 1923, iii, 370.

from one species to the related ones. This is exemplified by observations on crystalline hemoglobins.[25]

CONCLUSIONS.

The results of the partial saturation of precipitins with antigens related in derivation to the homologous one give no conclusive evidence of the regular existence in a single immune serum of multiple antibodies which act specifically on various chemical groups of the antigenic proteins. It seems possible to explain at least a part of the facts by the assumption that a single antibody will react to different degrees with several similar substances.

By the partial absorption of hemagglutinins with heterologous blood, specific fractions were obtained. By such means one may readily differentiate the blood of related species, even when precipitins show but little difference.

The peculiarities in specificity manifested by precipitinogens and agglutinogens suggest an essential difference in the chemical structures which determine the specificity of the two kinds of antigens.

[25]Reichert, E. T., and Brown, A. P., Carnegie Institution of Washington, Publication No. 116, 1909. Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, 1923–24, vi, 131.

THE EPIDEMIOLOGY OF A RABBIT RESPIRATORY INFECTION.

IV. Susceptibility of Rabbits to Spontaneous Snuffles.

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In previous communications we have pointed out that cultures made from the nasal passages of all rabbits affected with snuffles and of about 60 per cent of the so called normal stock rabbits at The Rockefeller Institute show *Bacterium lepisepticum*.[1,2] These findings led us to look upon this microorganism as the probable incitant of snuffles and to attempt to establish relationship between the presence of the bacterium in the nasal passages and the onset of the clinical symptoms. We had in mind also to determine whether accessory influences affect the susceptibility of rabbits to this disease.

Dr. Brown, Dr. Pearce, and Dr. Van Allen placed at our disposal 80 rabbits which were being used for experimental purposes.[3,4] The series contained normal, operated, and inoculated animals. Our method was to make cultures at frequent intervals of the nasal secretions of these rabbits in order to be able to compare the bacteriological findings present before spontaneous snuffles appeared with those as it developed. The swab technique employed has already been described.[1,2]

Spontaneous Clinical Snuffles.

Group 1. Normal Rabbits.—The nasal flora of twenty-two so called normal or control rabbits was determined at short intervals

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 843.

[2] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 857.

[3] Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 476.

[4] Pearce, L., and Van Allen, C. M., *Tr. Assn. Am. Phys.*, 1923, xxxviii, 315.

for periods of 1 to 4 months. During this time, four of these animals developed snuffles. Table I shows the bacteriological findings in the four affected rabbits.[5]

In each instance, *Bacterium lepisepticum* was present preceding as well as during the attack of snuffles. In one instance (Rabbit 23) the organism predominated in cultures at least for 1 month before

TABLE I.

Spontaneous Snuffles in Normal Rabbits.

Rabbit 23.				Rabbit 139.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*	Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 19	0	∞	0	Oct. 3	0	0	∞
" 26	0	∞	0	" 5	+	+	++
Oct. 17	++	∞	0	" 8	++	++	++
				" 11	++	++	++
				Nov. 8 Autopsy.	++	0*	++*
Rabbit 144.				**Rabbit 176.**			
Oct. 3	0	0	++	Oct. 17	0	0	∞
" 5	0	0	++	Nov. 8	0	0	∞
" 8	0	++	++	Dec. 12	0	0	0
" 11	+	∞	0	Jan. 29	+	∞	0
Nov. 8	++	∞	0	Mar. 8	+	∞	0
Autopsy.	++						

In thetables under Snuffles 0 indicates no snuffles; ±, questionable snuffles; +, moderate snuffles; ++, severe snuffles. Under *Bacterium lepisepticum* and *B. bronchisepticus* 0 indicates no colonies; +, 1 to 10 colonies; ++, 10 to 100 colonies; ∞ more than 100 colonies.

* Old plates inhibited. Growth of *Bacterium lepisepticum.* See Webster.[1]

the onset of symptoms. In another instance (Rabbit 139) the appearance of the bacterium coincided with the onset of symptoms

[5] Observations on the other animals of this series have been described already.[2] It was noted at that time that a certain number of them showed symptoms of chronic snuffles throughout the period of observation while others were clinically normal; that all those affected with snuffles carried *Bacterium lepisepticum* as the predominating organism in their nasal passages; and that 60 per cent of the normal rabbits carried *Bacterium lepisepticum* at one time or another.

and increased in number with the increase of the symptoms. In still another animal (Rabbit 144) the organism, previously absent, was found in large numbers a few days preceding the symptoms, while in Rabbit 176 the appearance of large numbers of the bacterium and the onset of the snuffles coincided.

Group 2. Operated, Uninoculated Rabbits.—This group contains five rabbits, thyroidectomized or thymectomized during the 3 months period of observation. Following operation, two of these animals developed snuffles. Here again the coincidence of the appearance of *Bacterium lepisepticum* and of the symptoms was striking. In one instance, the bacterium was cultured in large numbers from the left naris just as a left-sided discharge appeared. The right side at this time was free both from snuffles and *Bacterium lepisepticum*. There had previously been three negative cultures covering a period of 1 month. The other rabbit suddenly showed the bacterium in the nares and developed snuffles after five negative cultures over a 5 weeks period.

Group 3. Inoculated Rabbits.—The nineteen rabbits in this group were inoculated with *Treponema pallidum* or with a malignant rabbit tumor during the 3 to 5 months period of observation. Eight of the animals developed snuffles within this time.

In every instance *Bacterium lepisepticum* was present in the nasal passages either before or after the inoculation and always before the onset of the snuffles. Rabbits 44 and 130 yielded positive cultures 2 months and Rabbits 10 and 29 about 3 weeks before symptoms appeared, while Rabbits 36 and 122 showed the organism and clinical symptoms practically concurrently.

Group 4. Operated and Inoculated Rabbits.—Thirty-four rabbits which had been thyroidectomized or thymectomized and then inoculated with either *Treponema pallidum* or with a malignant rabbit tumor were observed for 5 months during which time thirteen developed spontaneous snuffles (Table II). Rabbits 22, 125, 32, 30, and 43 showed *Bacterium lepisepticum* for 1 to 4 months prior to the onset of symptoms and Rabbits 19, 130, and 179 less than 1 week before them. On the other hand, Rabbits 41, 126, 131, and 137 presented questionable symptoms and occasional positive cultures for varying periods of time, following which severe snuffles developed

TABLE II.

Spontaneous Snuffles in Operated and Inoculated Rabbits.

Rabbit 19.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 19	0	∞	0
" 26	++	∞	0
Oct. 17	++	∞	0
Nov. 2	Operation.		
" 9	++	∞	0
" 15	Inoculation.		
Dec. 17	0	0	∞
Jan. 20	0	0	++
Autopsy.			

Rabbit 22.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 19	0	++	0
" 26	0	++	+
Oct. 17	0	0	++
" 29	Operation.		
Nov. 12	0	0	++
" 15	Inoculation.		
Dec. 20	0	++	0
Jan. 15	++	∞	0
Autopsy.			

Rabbit 30.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 20	0	++	0
" 26	0	++	0
Oct. 17	0	∞	0
" 26	Operation.		
Nov. 12	++	∞	0
" 15	Inoculation.		
Dec. 14	±	++	++
Jan. 15	0	∞	0
Autopsy.			

Rabbit 32.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 20	0	∞	0
" 26	0	+	++
Oct. 17	0	0	++
" 26	Operation.		
Nov. 8	++	∞	0
" 15	Inoculation.		
Dec. 20	++	∞	0
Jan. 15	++	∞	0
Autopsy.			

Rabbit 41.			
Sept. 20	0	0	0
" 26	±	+	0
Oct. 19	±	0	0
Nov. 2	Operation.		
" 9	+	∞	0
" 15	Inoculation.		
Dec. 17	++	∞	0
Jan. 20	++	∞	0
Autopsy.			

Rabbit 43.			
Sept. 20	0	0	∞
" 26	0	0	∞
Oct. 19	0	++	++
" 26	Operation.		
Nov. 9	++	∞	0
" 15	Inoculation.		
Dec. 17	++	∞	0
" 26	++	∞	0
Autopsy.			

Rabbit 125.			
Oct. 3	0	0	0
" 5	0	0	0
" 8	0	∞	0
" 11	±	+	0
" 16	Operation.		
" 22	0	0	0
" 29	Inoculation.		
Nov. 17	0	∞	0
Dec. 14	0	+	0
Jan. 28	++	∞	0
Mar. 8	++	∞	0

Rabbit 126.			
Oct. 3	0	+	0
" 5	±	0	0
" 8	±	++	0
" 11	±	0	0
" 16	Operation.		
" 22	0	++	0
" 29	Inoculation.		
Nov. 8	+	∞	0
Dec. 14	±	∞	0
Jan. 28	++	∞	0
Mar. 8	+	∞	0

Rabbit 130.

Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Oct. 3	0	0	++
" 5	0	∞	0
Operation.			
" 8	++	∞	+
" 11	++	∞	0
" 22	++	∞	0
" 29	Inoculation.		
Nov. 8	++	∞	0
Dec. 14	++	∞	0
Jan. 29	++	∞	0
Mar. 8	++	∞	0

Rabbit 131.

Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Oct. 3	±	∞	0
" 5	±	0	++
" 8	0	0	++
" 15	Operation.		
" 22	0	0	∞
" 29	Inoculation.		
Nov. 8	++	∞	0
Dec. 11	++	∞	0
Jan. 29	++	++	0
Mar. 8	++	∞	0

Rabbit 137.

Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Oct. 3	0	0	0
" 5	0	++	0
" 8	0	0	0
" 11	0	0	0
" 16	Operation.		
" 22	0	0	0
" 29	Inoculation.		
Nov. 7	0	0	0
Dec. 12	++	∞	0
Jan. 28	++	∞	0
Mar. 8	+	∞	0

Rabbit 138.

Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Oct. 3	0	∞	0
" 5	0	∞	++
" 8	0	0	∞
" 11	0	0	++
" 16	Operation.		
" 22	0	0	++
" 29	Inoculation.		
Nov. 7	0	0	++
Dec. 12	L.		
	++	∞	0
	R.		
	0	0	0
Jan. 28	++	∞	0
Mar. 8	++	∞	0

Rabbit 179.

Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Oct. 17	0	0	∞
Operation.			
" 22	0	0	++
" 27	Inoculation.		
Nov. 7	0	0	++
Dec. 12	+	++	++
Jan. 28	++	∞	0
Mar. 8	++	∞	0

and the organism was recovered repeatedly in almost pure culture. Rabbit 138 presented a striking example (see Group 2, Rabbit 162) of early unilateral nasal discharge associated with *Bacterium lepisepticum* on the affected side only, followed later by an abundant discharge and large numbers of microorganisms on both sides.

In brief, then, throughout this entire series, *Bacterium lepisepticum* was present at some time in the nasal passages prior to the onset of snuffles and continued as the predominating organism throughout the clinical course of the disease.[6] A comparison of the percentage incidence of snuffles among the 22 control rabbits of Group 1 and the 58 operated or inoculated rabbits of other groups gives a 20 per cent incidence and one of 40 per cent respectively, thus indicating that the experimental procedures tended to impair resistance to this respiratory infection.

Disappearance of Clinical Snuffles.

Since it appears that the onset of spontaneous snuffles in rabbits is preceded by a longer or shorter period in which *Bacterium lepisepticum* is present in the nasal passages, and that this organism persists throughout the course of the disease, we undertook to ascertain by cultures whether, on spontaneous recovery, the bacterium could still be found. We selected from the above series of 80 rabbits those which during the 5 month period of observation had spontaneously recovered from snuffles. Table III summarizes the findings in six such instances.

Rabbit 19 developed severe snuffles 1 week or more after the appearance of *Bacterium lepisepticum;* 2 weeks later, the nasal discharge cleared up entirely and the organism was not again found. At autopsy not only were the nasal cultures negative but the mucous membranes seemed normal. In Rabbit 30, the bacterium was cultivated from the nares 6 weeks before the onset of snuffles. All symptoms disappeared 5 weeks later, but though at autopsy the nasal passages were normal, the organism was still present. Rabbit

[6] Data relating to the prevalence of *B. bronchisepticus* have been included in the tables because certain workers have considered this organism as the incitant of snuffles. Our studies provide no basis for this view but controvert it.

33 showed severe snuffles associated with *Bacterium lepisepticum* in nearly pure culture for at least 7 weeks. Both clinical symptoms and organisms then disappeared and at autopsy the nasal passages were normal and cultures were negative. Severe snuffles was associ-

TABLE III.

Recovered Cases.

Rabbit 19.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 19	0	∞	0
" 26	++	∞	0
Oct. 17	++	∞	0
Nov. 2	Operation.		
" 9	++	∞	0
" 15	Inoculation.		
Dec. 17	0	0	++
Jan. 20	0	0	++
Autopsy.			

Rabbit 30.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 20	±	++	0
" 26	0	++	0
Oct. 17	0	∞	0
" 26	Operation.		
Nov. 2	++	∞	0
" 15	Inoculation.		
Dec. 14	±	++	++
Jan. 15	0	++	0
Autopsy.			

Rabbit 33.			
Date.	Snuffles.	*Bacterium lepisepticum.*	*B. bronchisepticus.*
Sept. 20	++	∞	0
" 26	++	∞	0
Oct. 19	+	∞	0
" 26	Operation.		
Nov. 9	++	∞	+
" 15	Inoculation		
Dec. 17	0	0	+
Jan. 15	0	0	+
Autopsy.			

Rabbit 119.			
Oct. 3	+	∞	++
" 5	+	?	+
" 8	++	∞	0
" 15	Operation.		
" 22	++	∞	0
" 29	Inoculation.		
Nov. 7	0	0	++
Dec. 12	0	0	0
Jan. 28	0	0	+
Mar. 8	0	0	+

Rabbit 148.			
Oct. 3	0	0	0
" 8	+	∞	0
" 11	0	+	0
Nov. 8	0	0	0
" 14	0	0	0
Autopsy.			

Rabbit 177.			
Oct. 17	++	∞	0
	Operation.		
" 22	++	∞	0
" 29	Inoculation.		
Nov. 8	++	∞	0
Dec. 14	0	∞	0
Jan. 29	0	0	++
Mar. 8	0	0	++

ated with many organisms in Rabbits 119 and 117 during the first 3 weeks of observation, after which the symptoms cleared up and the bacterium could no longer be cultivated from the nasal passages. The presence of symptoms and organisms was limited to a few days in Rabbit 148.

In these six instances, then, the subsiding of symptoms or recovery from snuffles was associated with a diminution in number or a complete disappearance of the bacterium from the nasal passages.

DISCUSSION.

The data presented in this paper tend to support our previously expressed view that the presence in the nasal passages of *Bacterium lepisepticum* bears an etiological relationship to snuffles as it occurs at The Rockefeller Institute. The facts here brought out, taken together with observations previously published,[1,2] have led us to the conclusion that certain strains of the bacterium, widely distributed among the rabbits at The Rockefeller Institute, are of a grade of virulence too low to induce epidemic outbreaks of infection attended by septicemia and pleuropneumonia, but are yet sufficiently active to excite snuffles in a not inconsiderable percentage of the population.

According to the observations reported in this and preceding papers we may divide the rabbit population into three groups according to their resistance to strains of *Bacterium lepisepticum* that are of low virulence: (1) the resistant 20 per cent which do not offer a favorable medium for the growth of the bacterium; (2) the less resistant 40 per cent which carry the organism in their nasal passages without acquiring snuffles; and (3) the least resistant 40 per cent in which snuffles develops.

CONCLUSIONS.

The onset of spontaneous snuffles in rabbits at The Rockefeller Institute is preceded by the appearance in the nasal passages of *Bacterium lepisepticum*.

The active stages of snuffles infection are associated with the presence in these passages of large numbers of this bacterium.

Spontaneous recovery from snuffles is associated with a diminution in number or a disappearance of these bacteria from the nasal passages.

Various experimental procedures reduce the resistance of rabbits to spontaneous snuffles.

THE EPIDEMIOLOGY OF A RABBIT RESPIRATORY INFECTION.

V. Experimental Snuffles.

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A number of facts have been assembled to indicate that *Bacterium lepisepticum* is the incitant of snuffles as it occurs at The Rockefeller Institute.[1–3] We wish now to report in detail[4] the experimental induction of snuffles by the intranasal injection of this organism.

The experiments may be divided into two series; first, those in which regular stock rabbits were employed, which led to confusing results, and second, those in which carefully bred rabbits were used, which led to definite results.

For the first series of experiments, the rabbits were obtained from outside dealers. They were then placed in intimate contact with advanced cases of snuffles, or were inoculated intranasally, by various methods, with different strains of *Bacterium lepisepticum*, or were subjected to extremes of temperature or to operation prior to exposure to snuffles or inoculation with the organism just mentioned. As a whole, the results were difficult to interpret because the incidence of fresh infection among the experimental animals was small, while among the control uninoculated animals, spontaneous snuffles[3] was relatively frequent.

For example, four rabbits with clinical snuffles of 9 months duration were caged each one with a normal rabbit observed during the same period of time. Six similar normal rabbits were grouped two per cage as controls. To lower their

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 843.

[2] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 857.

[3] Webster, L. T., *J. Exp. Med.*, 1924, xl, 109.

[4] A preliminary announcement has appeared previously (Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 236).

resistance, some were then subjected to a temperature of 98.2°F. and some to 43.0° for 2 weeks. In the ensuing 3 months period of observation, two of the four normal rabbits in contact with the infected animals developed snuffles, but since four of the six controls came down with spontaneous snuffles, the significance of the experimental result remained in doubt.

In another similar experiment, the result was more favorable. Of four young rabbits, each placed in contact with an old snuffles rabbit and later subjected to bleeding or environmental change, three came down with snuffles while the controls remained normal throughout the 3 months period of observation.

Another experiment had to do with a group of eight rabbits in which an attempt was made to induce snuffles by introducing a culture of *Bacterium lepisepticum* into the nares on a cotton plug. In each case, removal of the plug after 24 hours was followed by a nasal discharge. In the controls it was serous in character and of short duration, while in the animals which had received the plugs containing *Bacterium lepisepticum*, a mucopurulent discharge developed which persisted from 4 days to 3 weeks.

Experiments such as these were of value only as they indicated the possibility that snuffles might be communicable, that environmental changes might influence the result, and that the appearance of snuffles is associated with the presence of *Bacterium lepisepticum* in the nasal passages.[5] They indicated further that variation in the susceptibility of the stock rabbits might be responsible for the confused results.

It was thought that part of this confusion might be avoided if the susceptibility-resistance factor was analyzed and controlled. Consequently it was decided to eliminate any specific acquired immunity of the rabbits to snuffles by establishing and maintaining a breeding room free of infection and free of *Bacterium lepisepticum*, the suspected etiological agent. This was done by choosing for breeding purposes rabbits which were clinically normal and with nasal passages free from the bacterium on at least three examinations. These animals received careful attention in a large, airy room, the ample cages being cleaned every day. At short intervals, the nasal passages of each animal were cultured and any carriers of *Bacterium lepisepticum* were removed. In this way we have maintained for 9 months a rabbit stock free from snuffles.[6]

[5] Further evidence of a similar nature is reported in the preceding paper.

[6] It may be of interest to note that a few of these rabbits carry *B. bronchisepticus* in their nasal passages.

Rabbits from this breeding room react in a characteristic and predictable manner to an intranasal inoculation of *Bacterium lepisepticum*. The technique of some experiments and the results follow.

Experiment 1.—A culture of *Bacterium lepisepticum* of low virulence was obtained from a rabbit showing snuffles and grown for 17 hours in broth, pH 7.4, containing ½ cc. of defibrinated rabbit blood. Five 800 gm. rabbits from the special breeding room received ¼ cc. of this culture into each nasal cavity by means of a blunt 2 cc. Luer syringe used as a medicine dropper. They were then placed in individual cages and observed for 3 months. Five uninoculated rabbits from the breeding room were kept under similar conditions as controls. Autopsies and bacteriological studies were employed to check the clinical findings.

Table I shows that three of the rabbits remained well but carried the bacilli in their nasal passages for more than 1 month and that two developed snuffles on the 1st and 2nd days after inoculation, the disease lasting 5 weeks and 3 months respectively and terminating in a fatal pleuropneumonia. The five uninoculated controls remained well and free of *Bacterium lepisepticum* throughout the period of observation.

Experiment 2.—A virulent culture of *Bacterium lepisepticum*, obtained from a rabbit by Dr. T. M. Rivers and grown for 18 hours in rabbit blood broth, was inoculated, as in Experiment 1, into the nares of six specially bred 800 gm. rabbits. These animals, together with five similar uninoculated rabbits, were then placed in separate cages and observed for 4 months. Autopsies and bacteriological studies were performed.

Table II shows that of the inoculated rabbits one remained well and carried the bacilli less than 1 month, another on the 1st day after inoculation developed snuffles which lasted less than 1 month, a third came down on the 1st day after inoculation with snuffles which lasted 6 weeks, while three developed pneumonia and died 3 to 4 days after inoculation.

Experiment 3.—Twelve 800 gm. rabbits from The Rockefeller Institute farm in New Jersey, free from snuffles and *Bacterium lepisepticum* as proven by four examinations covering a period of 2 weeks, received intranasally cultures of the same virulent Rivers strain as had been employed in the previous experiment. Each rabbit received ¼ cc. into each naris by means of a blunt 2 cc. Luer syringe. They were then caged separately and observed for 5 months. At autopsy the gross findings and bacteriology confirmed the clinical observations.

TABLE I.

Intranasal Inoculation of Bacterium lepisepticum (R 82) into Specially Bred Rabbits (Experiment 1).

Rabbit No.	Experimental procedure.	Clinical course.				Persistence of *Bacterium lepisepticum* in nasal passages.
		Immediate result.	Onset.	Duration.	Final result.	
101	*Bacterium lepisepticum* intranasally.	Well.	—	—	Well.	3 days to 2 mos.
107	" " "	Snuffles.	1st day.	3 mos.	Death 3rd mo. Pneumonia. Nares infection.	3 mos.
110	" " "	Well.	—	—	Well.	2 " +
113	" " "	Snuffles.	2nd day.	5 wks.	Death 5 wks. Pneumonia. Nares infection.	5 wks.
116	" " "	Well.	—	—	Well.	3 days to 2 mos.
Controls.						
1, 2, 3, 4, 5	—	"	—	—	"	—

TABLE II.

Intranasal Inoculation of Bacterium lepisepticum (Rivers Strain) into Specially Bred Rabbits (Experiment 2).

Rabbit No.	Experimental procedure.	Clinical course.				Persistence of *Bacterium lepisepticum* in nasal passages.
		Immediate result.	Onset.	Duration.	Final result.	
100	*Bacterium lepisepticum* intranasally.	Septicemia meningitis.	3rd day.	1 day.	Death 4th day. Septicemia. Meningitis. Nares infection.	4 days.
103	" " "	Snuffles.	1st "	1 wk.	Well.	2 wks.
106	" " "	Well.	—	—	"	1 wk.
109	" " "	Snuffles.	2nd day.	1 day.	Death 3rd day. Pneumonia. Nares infection.	3 days.
112	" " "	"	2nd "	1 "	" "	3 "
115	" " "	"	1st "	6 wks.	Well.	7 wks.
Controls.						
1, 2, 3, 4, 5	—	Well.	—	—	"	—

TABLE III.

Intranasal Inoculation of Bacterium lepisepticum (Rivers Strain) into Specially Bred Rabbits (Experiment 3).

Rabbit No.	Experimental procedure.	Clinical course.				Persistence of *Bacterium lepisepticum* in nasal passages.
		Immediate result.	Onset.	Duration.	Final result	
206	*Bacterium lepisepticum* intranasally.	Snuffles.	1st day.	5 mos. +	Snuffles.	5 mos. +
208	" " "	Well.	—	—	Well.	5 " +
212	" " "	Snuffles.	1st day.	6 days.	Death. Pneumonia. Nares infection.	7 days.
213	" " "	"	1st "	1 day.	Well.	5 mos. +
214	" " "	"	4th "	5 mos. +	Snuffles.	5 " +
215	" " "	"	1st "	1 mo.	Well.	5 " +
216	" " "	"	1st "	4 days.	Recurrence lasting 4 mos. +.	5 " +
217	" " "	"	1st "	4 "	Death. Pneumonia. Nares infection.	5 days.
219	" " "	"	3rd "	5 mos. +	Snuffles.	5 mos. +
220	" " "	"	1st "	5 " +	"	5 " +
221	" " "	"	1st "	5 " +	"	5 " +
222	" " "	"	1st "	5 days.	Death. Pneumonia. Nares infection.	6 days.

Table III shows that one rabbit remained well but carried the bacilli for over 5 months, that one showed 24 hours after inoculation a nasal discharge and matted paws lasting 1 day, seven developed snuffles which persisted 1 to 5 months, and three developed a nasal discharge and died with pneumonia 4 to 6 days after inoculation.

Experiment 4.—Seven 800 gm. rabbits from The Rockefeller Institute farm in New Jersey and twelve 700 gm. rabbits specially bred at the Institute in New York were used for this experiment. The New Jersey farm rabbits were free from snuffles and had shown no *Bacterium lepisepticum* in their nasal passages on three observations covering a period of 2 weeks. The specially bred rabbits had been examined several times while in the breeding room and were regularly free from snuffles and the bacterium.

Each rabbit received in each naris $\frac{1}{4}$ cc. of an 18 hour blood broth culture of *Bacterium lepisepticum* (Rivers strain) and was then placed in a separate cage for observation. Gross pathology and bacteriological studies at autopsy confirmed the clinical observations.

Of the specially bred rabbits, two remained well and carried the bacilli for less than 1 month, two developed snuffles on the 1st or 2nd day and recovered in less than 1 month, while eight rabbits showed a seropurulent nasal discharge and died of pneumonia 2 to 12 days after inoculation.

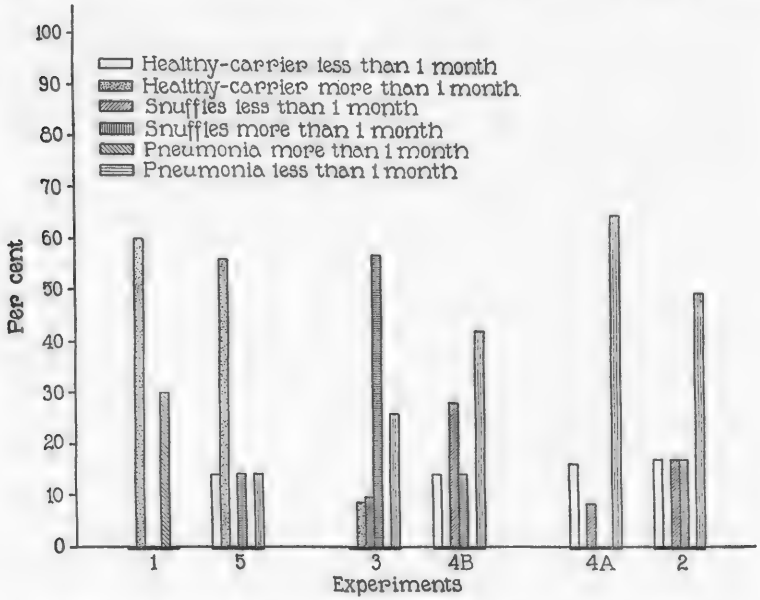
Of the New Jersey farm rabbits, one remained well and carried the bacilli less than 1 month, two developed snuffles on the 1st day after inoculation and recovered within 2 weeks, one on the 1st day developed snuffles which persisted more than 1 month, while three rabbits showed a slight nasal discharge, but developed pneumonia, and died 2 to 3 days after inoculation.

Experiment 5.—Seven 800 gm. rabbits from The Rockefeller Institute breeding room, with no history of snuffles and regularly free from *Bacterium lepisepticum* in the nasal passages, were inoculated into each naris with $\frac{1}{4}$ cc. of a 17 hour blood broth culture of the original Rivers strain and were placed in separate cages for observation.

One rabbit remained well and carried *Bacterium lepisepticum* less than 1 month, four remained well and carried the bacilli more than 1 month, one rabbit developed snuffles on the day following inoculation, which persisted more than 1 month, and one rabbit showed a slight nasal discharge and died of pneumonia 12 days after inoculation.

GENERAL RESULTS.

When the results of these five experiments are considered together, a number of interesting facts appear (Text-fig. 1). Thus, it is apparent from each experiment that normal rabbits react in different ways to an intranasal injection of *Bacterium lepisepticum* and that



TEXT-FIG. 1. Effect of intranasal inoculation of *Bacterium lepisepticum* into healthy young rabbits. The experimental data are arranged from left to right according to the severity of the response noted. Nos. 1 and 5 indicate low bacterial virulence; Nos. 3 and 4B indicate considerable host resistance; and Nos. 4A and 2 indicate high bacterial virulence.

this reaction indicates varying degrees of susceptibility. Therefore we have divided the rabbits into six categories according to their response, progressing from the most resistant to the most susceptible. (1) In the most resistant animals, the bacterium remains in the nasal passages for a few days without producing any noticeable disturbance; (2) in a more susceptible group, the bacterium remains in

the nasal passages for a period of months, in their case also without producing clinical symptoms; (3) in still more susceptible animals, clinical snuffles results, followed by recovery in a few days; (4) in yet others, chronic snuffles results and persists over a period of months; while the highly susceptible rabbits either (5) die of pneumonia and septicemia after a period of weeks or (6) die of pneumonia within a few days of the inoculation.

It is furthermore apparent that different strains of *Bacterium lepisepticum* and, indeed, different cultures of a given strain, vary in pathogenicity or virulence. Inoculations with Strain R 82 (Experiment 1) and the unpassed Rivers strain (Experiment 5) yielded a relatively great number of healthy carriers and snuffles cases with few pneumonia fatalities, while the virulent Rivers strain had result in few carriers and cases of snuffles, but a large number of fatalities from pneumonia.

DISCUSSION.

In the preceding pages it has been shown that the mere instillation of a virulent culture of *Bacterium lepisepticum* into the external nares of rabbits bred under circumstances of protection from infection with snuffles usually suffices, first, to set up an endemic carrier state, and second, to incite in certain animals a clinical condition identical with snuffles. It appears, therefore, that in the rabbit an active culture of *Bacterium lepisepticum* is not eliminated by the normal upper respiratory mucous membrane in the manner described by Bloomfield for certain pathogenic and non-pathogenic species in man.[7]

There is still confusion and difference of opinion in regard to the identity of the inciting microorganism of snuffles.[8] Recently Ferry and Hoskins[9] have concluded that the chronic form of the disease is induced by *Bacillus bronchisepticus* and the acute form by *Bacterium lepisepticum*. McCartney and Olitsky[10,11] who studied the

[7] Bloomfield, A. L., *Am. J. Med. Sc.*, 1922, clxiv, 854.

[8] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837.

[9] Ferry, N. S., and Hoskins, H. P., *J. Lab. and Clin. Med.*, 1919–20, v, 311.

[10] McCartney, J. E., and Olitsky, P. K., *J. Exp. Med.*, 1923, xxxviii, 599.

[11] Strain R 63 used by McCartney and Olitsky in their experiments had received eleven direct intrapleural passages but had not increased in virulence.

disease in stock rabbits state that *Bacterium lepisepticum* cannot be regarded as the sole inciting organism.

It is now obvious, we believe, that there are two sources for the lack of harmony in bacteriological experiments and findings: first, preexisting disease in the rabbits employed for testing, and second, the grade of virulence of the culture of *Bacterium lepisepticum* used for purposes of inoculation. Our experiments, carried out with rabbits bred with a view to protection from exposure to snuffles in any of its stages, and with strains of *Bacterium lepisepticum* of various degrees of virulence have led us to the conclusion that several degrees of natural resistance may be detected among groups of rabbits. According to these degrees of resistance a series of clinical and pathological effects is produced, either in course of ordinary exposure or experimentally, which may be classified as follows: (1) The carriers of *Bacterium lepisepticum* are rabbits of relatively high resistance which have been exposed to active cultures. (2) Moderately resistant rabbits tend on infection to come down with the local, nasal infection, snuffles. (3) Rabbits of maximum susceptibility on infection with *Bacterium lepisepticum* develop pneumonia associated with or without other manifestations of local or general infection.

In any given large group or community of rabbits, the quantitative ratio of the incidence of these several types of infection forms an index, if perhaps only a rough one, to the resistance of the rabbits and the virulence of the microbe.

CONCLUSIONS.

Rabbits were bred at The Rockefeller Institute with special relation to protection from exposure to *Bacterium lepisepticum*. These healthy rabbits were then submitted to intranasal instillation of a given dose of cultures of *Bacterium lepisepticum*. The results of the inoculation were various according to the natural degree of resistance of the individual animals.

The following graded effects could be distinguished: Some of the animals became (1) short time carriers of *Bacterium lepisepticum*; others (2) long time carriers of the same organism; some developed (3) local nasal infection (snuffles) of short duration; others (4) local

nasal infection (snuffles) of long duration; while yet others had (5) snuffles, followed by pneumonia and signs of general infection after a few weeks; and finally there were instances of (6) local nasal discharge attended by general infection and pneumonia after a few days.

The effects produced in any given healthy stock depend also upon the degree of virulence of *Bacterium lepisepticum* of which great differences exist among the same and different strains.

SURFACE TENSION OF SERUM.

IX. Time-Drop and Smallpox Vaccination.

By P. LECOMTE du NOÜY, Sc.D.

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(Received for publication, December 31, 1923.)

It has been shown in a previous paper[1] that the tensiometric method (measurement of the time-drop of the surface tension of a serum) affords a new means of determining the amount of antibodies contained in an immune serum. An endeavor was made to apply this method to the study of smallpox immunity in rabbits. Only a small number of cases were studied as there was no need of checking up the method itself, the results of which are always consistent and quite definite.

Ten rabbits were chosen, and the time-drop of their serum measured. In other words, 0.1 cc. of their serum was diluted 10,000 times in saline, and the surface tension of this solution was measured at 2 hour intervals with the du Noüy tensiometer. Six of these rabbits were found to yield data within the normal (their time-drop being: No. 1, 9 dynes; No. 2, 8.5 dynes; No. 3, 8.8 dynes; No. 4, 8.5 dynes; No. 5, 10.0 dynes; No. 6, 9.0 dynes). Four were not so satisfactory, but were kept nevertheless (No. 7, 10 dynes; No. 8, 11.5 dynes; No. 9, 12.0 dynes; No. 10, 11.0 dynes).

The method for measuring the surface tension was the same as the one used previously, but an improvement was made which allowed the solutions to remain absolutely undisturbed.[2]

Rabbits 1, 2, 7, and 8 were closely shaven on the loins, over an area of approximately 15 sq. cm. (a square of $1\frac{1}{2}$ by $1\frac{1}{2}$ inches). The vaccine was simply spread on this surface. 13 days later, they were bled, and the surface tension of the serum was measured according to the method just described. The results are shown in Table I.

[1] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.

[2] du Noüy, P. L., *J. Gen. Physiol.*, 1924, vi (in press).

According to the technique used by Dr. Noguchi, these animals were revaccinated after the 14th day in the same way, and none of them showed any reaction. This indicated that they were all immunized. The other six animals were vaccinated in a slightly more elaborate way.

Four equal areas about 1 by 2 inches square were closely shaven on the right loins, and different dilutions of the vaccine provided by Dr. Noguchi were applied: on the first area, pure vaccine; on the

TABLE I.

Time-Drop of 1/10,000 Solutions of Rabbit Sera before and after Vaccination.

		Normal.	13 days after vaccination.	Increase in time-drop.	
		dynes	*dynes*	*per cent*	
No. 1.	Initial value	64.0	75.0		
	After 2 hrs.	55.0	57.5		
	Time-drop	9.0	17.5	94	
" 2.	Initial value	63.0	76.0		
	After 2 hrs.	54.5	56.5		
	Time-drop	8.5	19.5	130	Immune.
" 7.	Initial value	66.0	73.5		
	After 2 hrs.	56.0	57.0		
	Time-drop	10.0	16.5	65	
" 8.	Initial value	69.5	76.0		
	After 2 hrs.	58.0	58.5		
	Time-drop	11.5	17.5	52	

second, vaccine diluted to 1/10 in saline; on the third, vaccine to 1/100; on the fourth, vaccine to 1/1,000. It is thus possible to know what is the smallest amount of vaccine that will produce a reaction. When the animals are revaccinated, the same procedure is followed, so that if immunity has not been complete, it is possible to measure, as it were, the acquired resistance by the shift of the reaction towards a higher concentration. Table II gives the value of the reaction of these rabbits, as well as the time-drop before and after vaccination.

As was to be expected, the sera with a relatively high initial time-drop (12, 11, 11.5, 10, and 10 dynes) did not show such a marked increase as the other five. It has been explained previously[1] that, this method being essentially non-specific, the values obtained for the time-drop are proportional to the amount of antibodies present, of

TABLE II.

Time-Drop of 1/10,000 Solutions of Rabbit Sera before and after Vaccination.

		Normal.	13 days after vaccination.	Reaction.	Increase in time-drop.	
		dynes	*dynes*		*per cent*	
No. 9.	Initial value	70.0	76.0	Good up to a dilution of 1/10.		
	After 2 hrs.	58.0	61.0			
	Time-drop	12.0	15.0		25	
" 10.	Initial value	68.0	75.5			
	After 2 hrs.	57.0	62.5			
	Time-drop	11.0	13.0	1/10	18	
" 3.	Initial value	76.0	77.0			
	After 2 hrs.	67.2	65.5			
	Time-drop	8.8	11.5	1/10	31	Immune.
" 4.	Initial value	73.5	76.5			
	After 2 hrs.	65.0	59.8			
	Time-drop	8.5	16.7	1/10	97	
" 5.	Initial value	75.5	77.0			
	After 2 hrs.	65.5	64.0			
	Time-drop	10.0	13.0	1/100	30	
" 6.	Initial value	75.5	77.0			
	After 2 hrs.	66.5	66.0			
	Time-drop	9.0	11.0	1/10	22	

whatever sort they be. If an animal considered as normal shows a time-drop above the normal (equal to or larger than 10 dynes), it is an indication that some antibodies exist already in its circulation.[3] In the case of rabbits, they may be consequent on the disease called snuffles, and will manifest their action on the time-drop when the animal is just recovering from a slight attack, or even during the

[3] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxviii, 87.

period of incubation. The presence of two kinds of antibodies does not seem to result in a summation of their effect on surface tension. Since the maximum drop thus far observed approximated 19 dynes, it is easy to understand that, when the value of the normal drop is above 10 dynes, the percentage increase will of necessity be limited by this maximum which is not subject to a shift. But, when all has been said, the considerable increase in the values of the time-drop in these ten instances, as well as the uninterrupted series of positive results obtained during a period of over 16 months, with a number of different antigens, seems to confirm definitely the value of the method. However, the nature of this phenomenon is still obscure. While the absolute value of the surface tension after 2 hours is decidedly decreased in certain cases (Experiments 3 and 6), in some others it is not, but is even increased, so that the increase in time-drop which was taken as a criterion of the presence of antibodies may depend on the increase of the initial value of the surface tension after immunization, or on the decrease of this value after 2 hours, or both. The time-drop itself is invariably increased. Further experiments will be made in order to try to elucidate this point.

SURFACE TENSION OF SERUM.

X. On the Thickness of the Monomolecular Layer of Serum.

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(Received for publication, March 21, 1924.)

INTRODUCTION.

The remarkable, fundamental studies of Lord Rayleigh,[1] Pockels,[2] and Devaux[3] opened a new field to physicists for the measurement of the dimensions of certain molecules. They were followed by those of Marcelin[4] and of Langmuir[5] who brought the subject into the limelight in a paper in which Devaux's experiments are reproduced, and all his assumptions and results confirmed. Devaux extended the work of Miss Pockels and of Lord Rayleigh, and developed experimental methods for the study of oil films which Langmuir quotes as being "beautiful in their simplicity and remarkable in the clearness with which they demonstrate the existence of monomolecular oil films." The experiments and calculations by which Devaux and Langmuir reached the dimensions of the molecules of oils, fats, or waxes are very simple. A known weight of the substance is dissolved in benzene. A drop of this solution is placed on water. It spreads, and the solvent (benzene) evaporates and leaves the monolayer of oil. (For convenience, the writer proposes the term "monolayer" instead of monomolecular layer.) The area covered by this layer is measured and, as the weight of the drop is known, the weight of oil divided by the total area gives its weight per square centimeter; hence, when the specific gravity of the substance is known, the thickness of the layer is readily obtained. If the specific gravity were equal to 1, the thickness in centimeters would evidently be equal to the number of grams per unit of surface (square centimeters). Ob-

[1] Lord Rayleigh, *Phil. Mag.*, 1899, xlviii, 331.

[2] Pockels, A., *Nature*, 1891, xliii, 437.

[3] Professor Devaux published a large number of papers between 1903 and 1914. A review of his work was published in the Annual Report of the Smithsonian Institute for 1913, page 261. Subsequent papers are: Devaux, H., *Soc. Franc. Phys.*, 1914, lv, 3; lvii, 3; and quite recently, *J. Phys. et Radium*, 1923, iv, series 6, 293.

[4] Marcelin, A., *Ann. Phys.*, 1914, i, 19.

[5] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

viously, this method rests entirely on the possibility of obtaining a monolayer, on the experimental evidence of its existence, and on the method devised to measure its area. The problem was relatively simple as long as non-soluble substances were used, since all the molecules remained at the surface of the water. But when soluble substances are dealt with, the problem is totally different. However, one kind of soluble substances (certain biological colloids) has been known to behave in a peculiar way on account of their lowering action on the surface energy of the solvent. The writer has shown[6,7] that an extremely small quantity of these substances was sufficient to lower substantially the surface tension of water, in function of time, and moreover that, at a certain critical dilution, the value of the "time-drop" (difference between the initial value and the value after 2 hours) reached a maximum. The discovery and study of this phenomenon was made possible by an instrument described previously.[8] In order to account for this maximum, the assumption was made that, at this concentration, a monolayer was formed. Subsequently, this criterion of the existence of a monolayer was supported by experimental evidence.[9]

The purpose of the present paper is to attempt to calculate the length of some colloidal molecules, on the basis of the experiments and the assumption just described.

EXPERIMENTAL.

The method for measuring the surface tension was the same as that used previously, but an improvement was made which allowed the solutions to remain absolutely undisturbed. This improvement is described in another paper.[10]

An attempt was first made to calculate the thickness of the monolayer of blood serum and serum proteins. For this purpose, a series of preliminary experiments was required to ascertain (1) the exact concentration at which the maximum drop took place; (2) whether the serum molecules were adsorbed on the glass as well as in the surface layer; (3) the specific gravity of *dry* proteins; and (4) the per cent of proteins in liquid serum.

[6] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.
[7] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.
[8] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.
[9] du Noüy, P. L., *J. Exp. Med.*, 1924, xxxix, 717.
[10] du Noüy, P. L., *J. Gen. Physiol.*, 1923–24, vi, 625.

1. Maximum Drop.—A series of very careful measurements was made with the technique heretofore described,[6,7,9,11–13] using the standard watch-glasses and 2 cc. of solution. The dilutions were chosen to cover a certain range on both sides of the concentration at which the maximum time-drop had been thought to occur; namely, 1/10,000. Thus, the series of concentrations studied was: 1/7,000, 1/8,000, 1/8,500 1/9,000, 1/9,500, 1/10,000, 1/10,500, 1/11,000, 1/11,500, 1/12,000, and 1/12,500. The results obtained with the rabbit serum used are given in Table I and Text-figs. 1 and 2. It will be observed that the initial values were quite constant, and that the value of the maximum time-drop depends only on the value after 2 hours. This observation gives considerable weight to the value of the quantity termed time-drop, as a criterion of the existence of a monolayer.

TABLE I.

Surface Tension and Time-Drop of the Surface Tension of Serum 242. Temperature 23°C.

December 28, 1923.

Concentration		1/8,000	1/8,500	1/9,000	1/9,500	1/10,000	1/10,500	1/11,000	1/11,500	1/12,000	1/12,500
Dynes.	Initial value	75.0	75.0	75.0	75.0	75.5	75.5	75.5	75.5	75.5	76.0
	After 2 hrs.	66.0	66.5	66.0	63.5	65.5	61.0	60.0	65.0	65.5	66.0
Time-drop		9.0	8.5	9.0	11.5	10.0	14.5	15.5	10.5	10.0	10.0

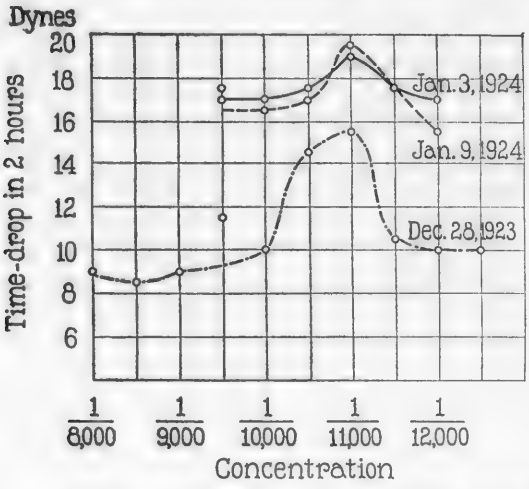
These experiments were repeated 5 and 11 days later with the same serum, under the same conditions. The results will be found in Table II and Text-figs. 1 and 3, and Table III and Text-figs. 1 and 4.

These tables show that the maximum drop of this serum always occurred at a concentration of 1/11,000, when diluted with saline solution (NaCl 0.9 per cent).

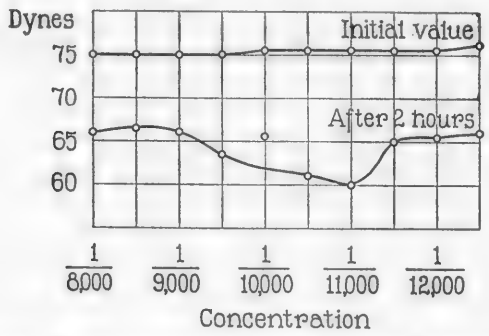
2. Are Serum Molecules Adsorbed on the Glass?—In order to answer this question, it was only necessary to observe the shift of the maximum drop as a function of the concentration in vessels differing

[11] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.
[12] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxviii, 87.
[13] du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 129.

from our standard watch-glasses. As has been pointed out in a previous paper,[14] the place of the maximum drop depends on the



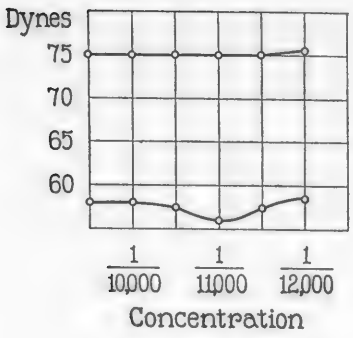
TEXT-FIG. 1. Maxima of time-drop of serum solutions in watch-glasses.



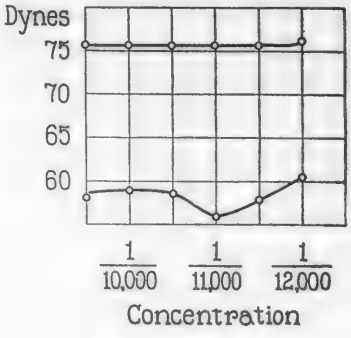
TEXT-FIG. 2. Surface tension of serum solutions in watch-glasses.

ratio $\frac{\text{Surface}}{\text{Volume}}$ of the vessels. The same amount of liquid, say 2 cc., if allowed to cover an area twice as large, will not be able to build

[14] du Noüy, P. L., *J. Exp. Med.*, 1924, xxxix, 37.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Surface tension of serum solutions in watch-glasses. Initial value and value after 2 hours.

TEXT-FIG. 4. Surface tension of serum solutions in watch-glasses. Initial value and value after 2 hours.

TABLE II.

Surface Tension and Time-Drop of the Surface Tension of Serum 242. Temperature 23°C.

January 2, 1924.

Concentration		1/9,500	1/10,000	1/10,500	1/11,000	1/11,500	1/12,000
Dynes.	Initial value	75.0	75.0	75.0	75.0	75.0	75.5
	After 2 hrs.	58.0	58.0	57.5	56.0	57.5	58.5
Time-drop		17.0	17.0	17.5	19.0	17.5	17.0

TABLE III.

Surface Tension and Time-Drop of the Surface Tension of Serum 242. Temperature 23°C.

January 8, 1924.

Concentration		1/9,500	1/10,000	1/10,500	1/11,000	1/11,500	1/12,000
Dynes.	Initial value	75.5	75.5	75.5	75.5	75.5	76.0
	After 2 hrs.	58.0	59.0	58.5	56.0	58.0	60.5
Time-drop		17.5	16.5	17.0	19.5	17.5	15.5

up a continuous monomolecular film and the number of molecules, *i.e.* the concentration, will have to be increased; in other words, the maximum will be shifted toward the higher concentrations. The contrary will happen if the amount of liquid is increased with little change in the surface. The ratio $\frac{S}{V}$ of the standard watch-glasses was very nearly $\frac{26.4}{2} = 13.2$, when the free surface, plus the surface of the glass wet by the solutions, was taken into account. The bottom of the watch-glass is a segment of a sphere whose radius is 7.2 cm. Its surface equals 13.33 when the depth of the liquid is 0.3 cm. Two series of experiments were made; in the first, the volume was unchanged (2 cc.) and the surface was increased, making the ratio $\frac{S}{V}$ larger (a shift toward the higher concentration was expected); and in the second, the surface was little changed and the volume increased fourfold, so as to obtain a ratio $\frac{S}{V}$ smaller and a shift toward the lower concentrations.

First Series.—The surface of the glass was considerably increased by the addition of 500 small beads to the bottom of the watch-glass.

These beads were, of course, cleaned carefully according to the technique described previously.[7,12] 50 of these glass beads were measured with a micrometer caliper, in order to obtain a mean value for their diameter. The sum was 47.68 which, divided by 50, gives a mean diameter of 0.952 mm. The surface of such a bead was 0.02847 sq. cm. which, multiplied by 500, gives 14.235 sq. cm. This surface, added to the existing area of glass (13.33 sq. cm.), brings it up to 27.56 sq. cm. By adding the free surface of the liquid (13.08 sq.cm.), we obtain for the total surface of adsorption $S' = 40.64$ sq. cm. The ratio $\frac{S'}{V}$ then becomes $\frac{40.64}{2} = 20.32$. The concentration at which the maximum drop should occur, if adsorption takes place on the glass, is related to the concentration at which the maximum occurs without the beads ($C = 1/11{,}000$) by the following equation, the volume of the liquid being unchanged: $\frac{S'}{S} = \frac{C'}{C}$ (C and C' being the concentrations). The value of C' is found to be very nearly equal to 1/7,000. Hence, in this case, the maximum drop should occur at 1/7,000, if adsorption takes place on the glass, and at 1/11,000, if it does not. Two control experiments were always carried on at the same time, in watch-glasses without beads. Control 1 was

made with the same solution; Control 2 with another series of solutions made separately, but from the same serum. The results are given in Table IV and Text-figs. 5 to 8. Only one table is published, as the text-figures express the phenomena in a clearer and shorter way.

TABLE IV.

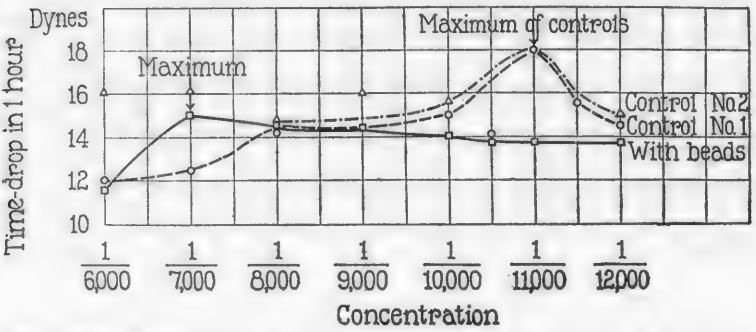
Surface Tension and Time-Drop of Serum Solutions in Saline in Standard Watch-Glasses.

Serum 232.

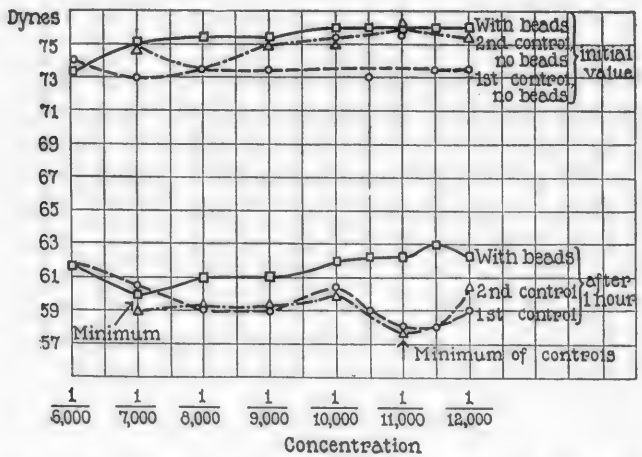
Concentration	1/6,000	1/7,000	1/8,000	1/9,000	1/10,000	1/10,500	1/11,000	1/11,500	1/12,000
With 500 glass beads in watch-glasses.									
Initial value	73.5	75.0	75.5	75.5	76.0	76.0	76.0	76.0	76.0
Value after 1 hr.	61.8	60.0	61.0	61.0	62.0	62.2	62.2	63.0	62.2
Time-drop (1 ")	11.7	15.0	14.5	14.5	14.0	13.8	13.8	13.0	13.8
Value after 2 hrs.	60.5	59.0	60.0	60.5	60.5	61.8	61.8	61.8	61.0
Time-drop (2 ")	13.0	16.0	15.5	15.0	15.5	14.2	14.2	14.2	15.0
Control 1, without beads.									
Initial value	74.0	73.0	73.5	73.5	75.5	73.0	76.0	73.5	73.5
Value after 1 hr.	62.0	60.5	59.0	59.0	60.5	59.0	58.0	58.0	59.0
Time-drop (1 ")	12.0	12.5	14.5	14.5	15.0	14.0	18.0	15.5	14.5
Value after 3 hrs.	60.0	58.0	58.0	58.0	58.5	58.5	57.5	57.5	58.0
Time-drop (3 ")	14.0	15.0	15.5	15.5	17.5	15.0	18.5	17.0	15.5
Control 2, without beads.									
Initial value		75.0	74.5	75.0	75.5		76.0		75.5
Value after 1 hr.		59.0	59.0	59.0	60.0		58.0		60.5
Time-drop (1 ")		16.0	14.5	16.0	15.5		18.0		15.0
Value after 3 hrs.		58.5	58.5	58.5	58.5		57.5		59.0
Time-drop (3 ")		16.5	15.0	16.5	17.0		18.5		14.5

Although the differences are small, the maximum is undoubtedly shifted from 1/11,000 to 1/7,000 by the presence of the beads, indicating that adsorption takes place on the glass. As pointed out above, the time-drop in this case can be replaced by the value after 2 hours. The initial value is always near 75 dynes, and is represented by a straight line, in a perfect experiment. In one case (Serum 252), the maximum with beads was at 1/9,000. However, this does not

affect the conclusions, as it may be due to some experimental error and the essential fact, namely the absence of a maximum at 1/11,000,



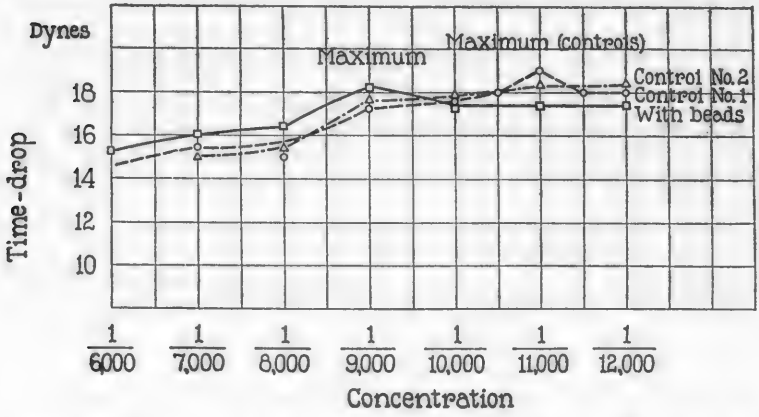
TEXT-FIG. 5. Time-drop of serum solutions in watch-glasses with beads and without beads (controls). Experiment made in order to show that adsorption takes place on the glass.



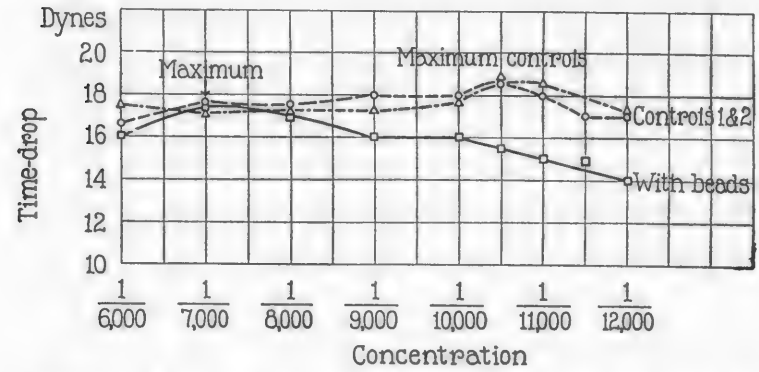
TEXT-FIG. 6. Surface tension of serum solutions in watch-glasses with beads and without beads (controls). Experiment made in order to show that adsorption takes place on the glass.

is quite clear. These experiments are extremely delicate and require great care. The monolayer is very fragile, and the difference

is relatively small between the number of adsorbed molecules at 1/10,000 and at 1/11,000. What is actually measured at the second



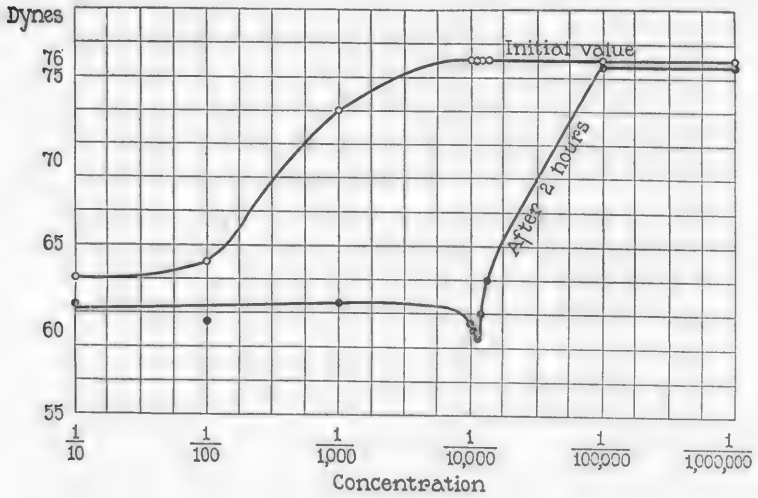
TEXT-FIG. 7. Time-drop of serum solutions in watch-glasses with beads and without beads (controls). Experiment made in order to show that adsorption takes place on the glass.



TEXT-FIG. 8. Time-drop of serum solutions in watch-glasses with beads and without beads (controls). Experiment made in order to show that adsorption takes place on the glass.

reading, after 1 or 2 hours, is the difference in the stress between three different sorts of layers: (1) the perfect monolayer, with the

minimum stress at a well defined dilution; (2) the monolayer underlaid or reinforced by patches of another layer of molecules; and (3) the residue of the continuous monolayer when it is broken up into floating tables, with free water canals between them, like the floe of an ice-field after the break-up. On both sides of the perfect, continuous monolayer, the surface tension is slightly higher. But on the side of the higher concentration, its value is practically the same, from 1/11,000 to 1/10 (the smaller time-drop being due to the decreasing values of the initial surface tension), while on the



TEXT-FIG. 9. Surface tension of serum solutions. Initial values and values after 2 hours.

side of the lower concentration, its value increases rapidly. A set of curves representing the perfect phenomenon is given in Text-fig. 9. A thorough investigation of the surface tension of solutions on both sides of the critical value 1/11,000 is now being made in this laboratory.

Second Series.—In this series of measurements, the volume of liquid was changed, while the free surface remained the same and the glass surface was increased as little as possible. Of course, as the number of molecules was increased (four times, as 8 cc. were

used), lower concentrations had to be prepared, so that every molecule could find a place on the adsorbed layer, and no free molecules could exist in the bulk of the solution.

Petri dishes 4 cm. ± 0.05 cm. in diameter were chosen. The depth of the liquid, when the dishes were filled with 8 cc. of solution, was about 0.63 cm. The total surface of adsorption, including glass, was 33.1 sq. cm., and the surface of the liquid, as in the watch-glasses, 13.08 sq. cm. If it is assumed that there is adsorption on the glass, the ratio, $R' = \frac{S}{V}$, was equal to $\frac{33.1}{8} = 4.15$, roughly 4. In the other case (adsorption on the free surface only), it was equal to $\frac{13.08}{8} = 1.63$. In the first case ($R' = 4$), the maximum time-drop should take place at a concentration determined as follows (R = watch-glass ratio $\frac{26.4}{2} = 13.2$):

$$\frac{R'}{R} = \frac{C'}{C}, \text{ i. e. } \frac{4.0}{13.2} = \frac{\frac{1}{x}}{\frac{1}{11{,}000}}, \text{ whence } \frac{1}{x} = \frac{1}{35{,}000}$$

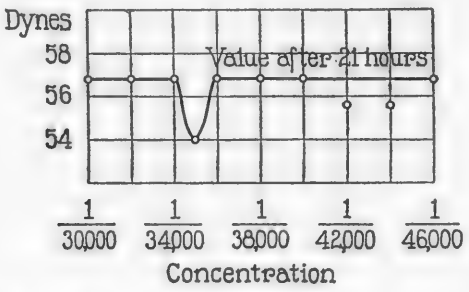
In the second case (no adsorption on the glass), the surface being the same as in the watch-glasses, and the volume V being increased fourfold, the concentration should be four times smaller; namely, 1/44,000. Hence, if there is adsorption on the glass, the maximum should occur at 1/35,000; if there is no adsorption, at 1/44,000. As the thickness of the liquid was much greater than in watch-glasses, more time was allowed to elapse between the two measurements of surface tension.

The results of the readings after 21 hours are given in Text-fig. 10. A sharp minimum is seen at 1/35,000, in conformity with our assumption. On account of the still greater difficulty of these measurements, as the surface of a larger mass of liquid will not remain immobile, the values of the initial surface tension were not quite constant, and only the values after 21 hours, on which rests the criterion of the existence of the monolayer, are published.

The two series of experiments reported above show that adsorption takes place on the glass as well as on the free surface. Moreover, as the place of the calculated maxima coincides satisfactorily with the concentration observed, it may be concluded that the orientation of the molecules is probably the same when adsorbed on the

glass and on the free surface. Otherwise, the occupied area would be different unless the molecules had undergone a rotation of 190° in a plane perpendicular to the surface. As it is more reasonable to think that the carboxyl groups are always drawn toward the water, it may be assumed that there is no change in orientation.

3. Specific Gravity of Anhydrous Proteins.—A sample of the serum used was dialyzed during 4 days, to deprive it of its salts. Then it was dried in a desiccator, and the resulting flakes were placed in different mixtures of benzene and chloroform. The specific gravity of the mixture in which the flakes floated without moving up or down was found to be 1.275, which was, therefore, the specific gravity of the proteins. Three sera gave the same value.



TEXT-FIG. 10. Surface tension of serum solutions in Petri dishes.

4. Percentage of Proteins in Rabbit Serum.—The percentage of proteins in the serum was determined by the Kjeldahl method (nitrogen titration) and found equal to 6.51 per cent. This figure is in good accord with the data published by Mathews[15] and Arthus[16] (total nitrogen, 1.080 per cent; non-protein nitrogen, 0.038 per cent).

Calculation of the Thickness of the Adsorbed Layer.—The calculation is made in the following way.[17]

[15] Mathews, A. P., Physiological chemistry, New York, 2nd edition, 1916, 461–463.

[16] Arthus, M., Précis de chimie physiologique, Paris, 5th edition, 1908, 147.

[17] In all the following calculations, it has been assumed that the increase in thickness of the monolayer due to adsorbed sodium molecules was negligible.

The serum contains 6.51 per cent of proteins, and is diluted 11,000 times (maximum drop at 1/11,000). The final dilution of the dry substance is 1/168,000. 2 cc. of the solution are used in every watch-glass, that is $\frac{2}{168{,}000} = 0.0000119$ gm. of protein per water-glass. This weight of proteins (11.9×10^{-6} gm.) is spread evenly, according to the hypothesis, on an area of 26.4 sq. cm. (monolayer). Thus, the weight of proteins per sq. cm. will be $11.9 \times 10^{-6} : 26.4 = 4.51 \times 10^{-7}$ gm. If the specific gravity of these substances were

TABLE V.

Dimensions of Some Organic Molecules.

	Cross-section.	$\sqrt{\text{Cross-section.}}$	Length.	Ratio: $\frac{\text{Length}}{\sqrt{\text{Cross-section}}}$
	sq. cm.	*cm.*	*cm.*	
Palmitic acid	21×10^{-16}	4.6×10^{-8}	24.0×10^{-8}	5.20
Tristearin	66×10^{-16}	8.1×10^{-8}	25.0×10^{-8}	3.10
Oleic acid	46×10^{-16}	6.8×10^{-8}	11.2×10^{-8}	1.65
Triolein	126×10^{-16}	11.2×10^{-8}	13.0×10^{-8}	1.16
Cetyl palmitate	23×10^{-16}	4.8×10^{-8}	41.0×10^{-8}	8.55
Myricyl alcohol	27×10^{-16}	5.2×10^{-8}	41.0×10^{-8}	7.90
Egg albumin, crystalline; water solution pH 6.8; assumed molecular weight 34,000 (du Noüy)	930×10^{-16}	29.2×10^{-8}	52.8×10^{-8}	1.70

equal to 1, the thickness of the monolayer would be 4.51×10^{-7} cm. But it is equal to 1.275; consequently, 4.51×10^{-7} must be divided by 1.275, and we obtain 3.54×10^{-7} cm., or 3.54 $\mu\mu$ for the thickness of the monolayer of total proteins in this serum. This figure represents the *mean* value of the length of albumin and globulin molecules. The thickness of the monolayer of a total serum (including amino acids) was found to be 4.05×10^{-7} cm. The determination of the length of serum albumin and serum globulin molecules separately is now being undertaken in this laboratory.

It may be of interest to mention the fact that the same method applied to the determination of the size of fresh egg albumin (crystalline) in water, with a pH of 6.8, prepared by Dr. Baker in this laboratory, yielded the following results: length, 5.28×10^{-7} or 52.8×10^{-8} cm.; and the $\sqrt{\text{cross-section}}$ (which is the width or very nearly the average diameter of the molecule), if Sörensen's probable value of 34,000 for the molecular weight is taken, was found to be 2.92×10^{-7} cm. If the molecular weight is taken as 15,703,[18] the $\sqrt{\text{cross-section}}$ becomes 2.0×10^{-7}.

To show how these dimensions compare with those of some insoluble organic molecules, a few figures taken from Langmuir's[5] paper are given here, together with the writer's estimate of the size of the egg albumin molecule, and other data.

TABLE VI.

Diameter of Some Colloidal Particles (Metallic Colloids).

Gold	60–1,300 $\times 10^{-8}$ cm.
Silver	500–7,700 $\times 10^{-8}$ "
Platinum	440 $\times 10^{-8}$ "
Wave-length of violet light	4,000 $\times 10^{-8}$ "

DISCUSSION AND CRITICISM.

The value obtained by the method here outlined for the mean thickness of the monolayer of serum is exact. But whether this dimension represents the mean length of the individual molecules composing the serum depends on the value of the criterion for the existence of a monolayer at this concentration; namely, the minimum value of the surface tension after a certain time, or the maximum time-drop.[19] The dimensions of the albumin molecule, as given in

[18] Mathews, A. P., Physiological chemistry, New York, 2nd edition, 1916, 139.

[19] Since this paper was written, experiments carried out along identical lines with sodium oleate have shown beyond doubt that all the assumptions concerning the existence of the true solution, of the monolayer, and of the adsorption on the glass were correct. For the first time, it has been possible to measure accurately, not only the length, but also the two other dimensions of a molecule (Sodium oleate) (du Noüy, P. L., *Phil. Mag.*, 1924 (in press): *Science*, 1924 (in press)).

Table V, also depend on the correctness of this assumption. The experimental data in favor of it have already been mentioned. They are the occurrence of a maximum time-drop, corresponding to a well defined minimum value of the surface tension, and the decrease in the rate of evaporation of the solutions at a certain concentration, phenomena which strongly support the hypothesis of an organized layer at this concentration. Another fact can now be added, the clear shift of the maximum drop when the surface adsorption is increased. And finally should be mentioned the striking circumstance of the value of the ratio $\frac{\text{Surface}}{\text{Volume}}$ in the case of capillary vessels filled with cells, precisely of that order of magnitude which would require absolutely pure plasma, such as exists in the circulation, to build up a monolayer on the walls of the capillaries and cells.[14]

The value as evidence of the maximum time-drop, on which the writer originally based his assumption of the existence of a monolayer,[7] has been considerably increased by the discovery of the sharp minimum value of the surface tension after 2 hours, which a closer study of the phenomenon has revealed (Text-fig. 9). The weakness of the conception of the maximum drop had been its dependence upon the value of the initial surface tension. The later experiments have shown that it corresponds to an actual critical value of the surface tension after 2 hours, which can be readily explained by the formation of a monolayer at this concentration. Indeed, it is easy to understand how such a layer, in which no forces exist save those surrounding the upper part of the adsorbed molecules, will produce a minimum field of forces. It is only when the molecules are allowed to wander undisturbed from the bulk of the solution, and to distribute themselves on a perfectly free surface, that such a layer can be formed. Supposing that the solution is slightly shaken, or that some microscopic particle of dust floats at its surface, or simply that, for some unknown cause, a few molecules lie horizontally, instead of vertically, then the minimum surface tension will nevertheless be found at the same concentration exactly, presumably because the differences existing between the length and width of these molecules are so small (ratio, $\frac{\text{Length}}{\text{Width}} = 1.7$, in the case of albumin, as

shown in Table V). Yet under such circumstances the value of the tension may differ materially, as shown by experiments. It is quite obvious that, if the molecules were spherical, which is difficult to admit on a purely chemical basis, such changes would be impossible to account for, as their field of forces would be spherical, hence, identical in all directions.

All the preceding considerations are strongly in favor of the conception of the existence of a monolayer at a concentration of approximately 1/11,000 for rabbit serum (corresponding to a dilution of the proteins to 1/168,000), and at 1/117,500 for egg albumin (crystalline, pH 6.8). This gives 35.4×10^{-8} cm. for the mean value of the length of the molecules of proteins (albumin and globulin) of the rabbit serum, and about 53×10^{-8} cm. for the mean length of the egg albumin molecule. As the molecular weight of these substances is only assumed, the writer does not wish to lay emphasis on the values of the possible cross-section of these molecules. Experiments are under way in this laboratory to determine, by the method already described, the thickness of the monolayer of serum albumin and of serum globulin, and the differences which may exist between the molecules of normal and of immune serum.

It may be objected that the fatty acids and cholesterol contained in the serum play an important part in the time-drop. But it is very probable that the substances, which are normally not soluble in water, are part of the molecules adsorbed in the surface and have no independent action on the surface tension of the water. As a matter of fact, solutions of pure crystalline serum and egg albumins, and globulins, entirely devoid of fats, give practically the same values for the time-drop as the total serum (12 to 16 dynes).

SUMMARY.

An attempt was made to apply the assumption that a monolayer of serum exists at a certain dilution, in order to calculate the thickness of this layer or, that is to say, the mean value of one of the dimensions of the molecules of the serum proteins. The criterion taken for the existence of such a monolayer was the existence at a given concentration (1/11,000 for rabbit serum) of a maximum drop in the surface tension of serum solutions kept in watch-glasses.

A series of preliminary experiments showed:

1. That the maximum drop in 2 hours took place, for the material used, at a concentration of 1/11,000, and that it always corresponded to an absolute minimum value of the surface tension of the solution, this minimum being quite sharp and well defined.

2. That adsorption took place on the glass as well as on the free surface of the liquid, and that apparently the same part of the molecule, in both cases, was drawn toward the water.

3. That the specific gravity of the anhydrous proteins of the rabbit serum studied was 1.275, whence it followed, on the basis of 6.51 per cent protein content, that the mean thickness of the protein molecules was 35.4×10^{-8} cm. The same method applied to crystalline egg albumin, pH 6.8, in water, gave 52.8×10^{-8} cm. for the probable molecular length.